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Hypothalamic Control of Pituitary Function and Corpus Luteum Formation in the Rat.* (23101)

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Following bilateral hypothalamic lesions in the region of the ventro-medial nucleus in the rat, persistent cornification of the vaginal epithelium is observed in a large number of the rats which eventually become obese, as well as many of those failing to develop obesity. Interference with production of corpora lutea following destructive lesions in the anterior hypothalamus of the guinea pig has been described by Dey, Fisher, Berry and Ranson (1). Brookhart, Dey and Ranson(2) demonstrated that such ovaries, characterized by follicles only, became luteinized following administration of a single dose of pituitary luteinizing factor, substantiating the conclusion that this type of lesion interfered with pituitary secretion of luteinizing hormone. Hillarp(3) localized a center controlling luteinization in the rat in the anterior hypothalamic area immediately anterior and ventral to the paraventricular nucleus. Bilateral lesions in this area resulted in ovarian changes characterized by normal follicular development and abundant interstitial tissue but no corpora lutea.

It was the object of the present study to reinvestigate hypothalamic control of corpus luteum formation in the rat. As complete

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an endocrine study as practical was made to determine the functional state of the pituitary in the presence of such lesions, and replacement therapy with luteinizing (or interstitial cell stimulating) hormones was instituted.

Materials and methods. Female rats of the Sprague-Dawley strain, 80-90 days of age, were used. All animals were maintained on Diet I† *ad libitum,* with the exception of one group in which diet was restricted to reduce weight. Bilateral hypothalamic lesions were produced in 200 g rats using the Horsley-Clarke instrument adapted by Clark(4) for use on the rat. Under ether anesthesia, electrolytic lesions were made with a direct current of 1.8 milliamperes for 15 seconds, using a unipolar electrode introduced through a drill hole in the parietal bone. The lesions were placed ventral to the ventro-medial nucleus of the hypothalamus. Daily vaginal smears and weekly check of body weight were recorded for 5 months on operated rats as well as on a group of unoperated controls. Twenty-four hours prior to autopsy (5 months post-operative), metabolic rates were determined with a closed-circuit multiple respiration apparatus, patterned after that described by Kleiber(5).‡ All rats were fasted 4 hours and allowed to adjust to the apparatus for one hour prior to oxygen consumption measurements, which extended over a 2.5 hour period. The liters of dry oxygen, at standard temperature and pressure, consumed by each rat per hour were converted to calories per hour using 4.80 as the caloric equivalent. The calories per hour thus obtained were expressed per square meter of body surface using Benedict's formula(6) for the rat:

$$\text{area in } m^2 = \frac{9 \times \sqrt[3]{(\text{body wt in g})^2}}{10{,}000}.$$

To measure I^{131} uptake of the thyroid, 1 μc of I^{131} was given (intraperitoneally, 16 hours prior to autopsy) and activity of thyroid determined by the scintillation counter. Just prior to autopsy blood volume and total circulating red cell volume were determined by the Fe^{59} labeled cell dilution method(7). Following blood volume determination, the animals were killed with chloroform. Their specific gravity was measured by the method of volume displacement using a graduated cylinder containing water. The pituitary, thyroid, adrenals, thymus, ovaries, uterus and oviducts were carefully dissected and weighed. After weighing, the adrenals were fixed in neutral formalin, imbedded in gelatin, cut in frozen section and stained with Sudan black for demonstration of lipid distribution. Thyroids and ovaries were fixed in Bouin's fluid, imbedded in paraffin and stained with hematoxylin-eosin. The pituitaries were fixed in Zenker-formalin, weighed, imbedded in nitrocellulose, cut at 4 μ and stained with modified Mallory-Azan stain(8). The calvarium was cut away and the brain carefully lifted from the cranium, fixed in 95% alcohol, imbedded in paraffin and the hypothalamic area sectioned serially at 10 μ. Every 10th section was mounted and stained with thionin.

Results. Excessive increase in body weight began immediately in the majority of the 53 rats, in which bilateral lesions had been placed ventral to the ventro-medial nucleus. The animals which eventually became obese gained 2 to 8 g per day in the first week after operation. Of rats which became obese, most (80%) developed persistent cornification of the vaginal epithelium within 2 weeks following the operation. The remainder showed either persistent diestrus smear or normal cycles. Of the operated animals which did not become obese, ½ developed persistent cornification, and the remainder continued to show either a normal rhythm, or a persistent diestrus smear. Those animals which became obese were irritable and difficult to handle.

After 5 months, groups of 5 to 7 rats, representing each type of response, were autopsied, 1) obese showing constant vaginal cornification; 2) obese, showing normal cycles;

† Diet K (modified from McCollum's formula) consists of 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of vit A and 2.5 A.O.A.C. chick units of vit D per g of diet. This diet was supplemented by fresh lettuce twice weekly.

‡ We wish to thank Dr. E. S. Evans for determinations of metabolic rate.

TABLE I. Comparison of Body Size, Reproductive and Endocrine Systems of Rats with Hypothalamic Lesions. (Avg 5 rats/group.)

Category	Obese, constant vaginal cornification	Obese, normal cycles	Not obese, constant vaginal cornification	Unoperated controls, normal cycles
Body wt (g)	*414 ±12 †	467 ±30	248 ±10	287 ± 7
Specific gravity	* .98± .02	.98± .01	1.05± .01	1.04± .01
Red cell vol (cc/100 g body wt)	1.30± .11	1.37± .11	1.71± .10	1.68± .08
Thyroid wt (mg)	29 ± 3	24 ± 2	17 ± 2	22 ± 2
I^{131} uptake (%)	22.1 ± 4.8	14.6 ± 5.8	12.1 ± 1.9	7.2 ± 2.3
Stand. metabolic rate (Cal./m²/hr)	32.9 ± 3.1	30.1 ± 2.1	32.7 ± 1.7	30.8 ± .7
Adrenal wt (mg)	57 ± 2	62 ± 5	56 ± 3	60 ± 4
Thymus " "	150 ±34	125 ±21	123 ± 8	132 ± 12
Pituitary " "	14.1 ± 1.4	12.1 ± .4	12.5 ± .8	13.0 ± .8
Ovarian wt (mg)	43 ± 5	62 ±17	36 ± 2	81 ± 5
Follicles‡	s, m, l cysts	s, m, l	s, m, l cysts	s, m, l
Corpora lutea	0	Several	0	Several
Uterus + oviducts (mg)	800 ±53	598 ±38	818 ±52	654 ±119

* Two rats, whose body wt was reduced by restricting food, were not included in the average.

† Mean and stand. error.

‡ Small (s), medium (m), large (l); cysts, *i.e.* in excess of size of mature follicle.

3) not obese, showing constant vaginal cornification; 4) unoperated controls. The results of the various determinations made are summarized in the Table. Animals designated as obese weighed 60% more than the non-obese groups, and specific gravity of the obese rats was significantly less than that of the non-obese rat.

The ovaries of the animals showing persistent vaginal cornification contained no corpora lutea, although follicles were abundantly present. The interstitial cells of the ovary were epithelioid, appearing healthy except for an excess lipid content. The follicles were of all sizes, including several in excess of normal size; many were in different stages of atresia. Infolding of the granulosa was present in some of the largest or cystic follicles.

The ovaries of rats which had shown normal vaginal cycles contained corpora of different ages, also growing follicles and normal appearing epithelioid interstitial tissue. The weight of ovaries, which contained follicles, was considerably less than that of ovaries containing corpora lutea.

In obese rats with constant cornification, I^{131} uptake of the thyroid was 3 times that of controls. The thyroids of this group weighed only slightly more than the controls, but gave histological evidence of increased activity. There was no significant difference in standard metabolic rate of the 4 groups. This is in contrast to the findings of Greer(9), Bogdanove and Halmi(10), Ganong *et al.*(11) and D'Angelo and Traum(12) who found evidence of decreased TSH secretion following lesions in the hypothalamus.

Adrenal, thymus, pituitary weights and tail lengths were not different in the 4 groups. The total circulating red cell volume was low in the obese animals.§ As red cell volume is expressed in relation to body weight this low figure is undoubtedly attributable to adiposity. It is known that increase in blood volume accompanying fat deposition is less than that accompanying increase in lean body weight.¶

The pituitaries from animals showing constant vaginal cornification were distinguishable histologically from those of normal controls, even though normal rats were in different stages of the estrous cycle at time of autopsy. They were characterized by a decrease in number of normally granulated, deeply staining acidophils and by an increase in number of almost completely degranulated acidophils, which were indistinguishable from chromophobes except after careful examina-

§ Total circulating red cell volume of the controls was considerably less than that for adult rats of the Long-Evans strain(7).

¶ Garcia, J. F., USAEC, UCRL 3516.

tion. These cells differed from chromophobes by their larger size, by distinctness of cellular outlines, by enlarged negative image of the Golgi apparatus, as well as persistence of a few acidophilic granules. The pituitaries of animals with lesions also showed decreased numbers of basophils; those which remained were irregular in contour and were situated within cords and not in intimate contact with the blood vessels. Their nuclei were vesicular, they had prominent nucleoli and the Golgi apparatus was in close proximity to the nucleus. This distribution and morphology suggest that they are thyrotropic basophils, since gonadotropic basophils are oval or rounded in shape, without prominent nucleoli, and with the negative image of the Golgi apparatus situated definitely away from the nucleus. The location of gonadotropic basophils is also described as bordering the sinusoids not within the cellular cords. The decrease in the number of basophils was therefore possibly due to decrease of gonadotropic basophils. In rats which were obese, but with normal vaginal cycles, the pituitaries showed normal basophils of both thyrotropic and gonadotropic types. Some degranulation of acidophils was present but not to the same degree as in the animals showing constantly cornified vaginal smear.

Serial sections of the hypothalamic area were examined and the lesions were diagrammed using the schematic graphs of Krieg. It was found that of 10 animals examined, which showed constant vaginal cornification (5 obese and 5 not obese), the lesion lay ventral to the ventro-medial nucleus in 9 and ventral to the anterior nucleus in 1 (obese). Of 4 animals examined which were obese but maintained normal vaginal cycles (several crops of corpora present in the ovary), 3 had lesions placed more anteriorly than those in the constant estrous group (ventral to the anterior nucleus) and in 1 the lesion was more posterior (ventroposterior to the ventromedial nucleus).

Progesterone. According to Greer(13) rats showing obesity and constant vaginal cornification, following bilateral lesions in the rostral hypothalamus, could be made to cycle by administration of progesterone. For this reason, progesterone was given to 3 rats which had developed obesity and persistent vaginal cornification; one was given 0.5 mg of progesterone (the dose used by Greer), and 2 were given 5 mg, each dose given subcutaneously in cottonseed oil daily for 4 days. By 24 hours leucocytes had appeared in the vaginal smear and by 48-72 hours the smear was typical of diestrus. On the fifth day, the animals were autopsied and ovaries examined grossly and histologically. No corpora lutea were present and there was no evidence of luteinization of follicle walls. The incidence of follicular atresia was, however, definitely increased in all progesterone treated animals.

Chorionic Gonadotrophin. Five rats which became obese following operation and showed a persistent cornification of the vaginal smear, were given graded doses of chorionic gonadotrophin in the hope of inducing corpus luteum formation and ovulation. A total dose of 5, 10, 25, 50 or 150 rat units was given in divided doses subcutaneously for 4 days. On the third day of injection, leucocytes were present in the vaginal smear and by the fourth day the smear was typical of diestrus in those rats receiving 25 to 150 rat units, but remained cornified at the lower doses. The ovaries of rats given 25 rat units or more when observed on the fifth day, contained a single group of large, newly formed corpora lutea of approximately the same age. No luteinization was observed at the 2 lower doses. The oviducts were not distended. The interstitial tissue had become more normal in appearance; the excess lipid characteristic in the interstitial tissue of untreated animals had disappeared. The uterine mucosa of animals given 150 rat units of chorionic gonadotrophin showed very active glands, with wide openings. Such functional change in uterine mucosa would not be expected unless lactogenic hormone had been secreted from the pituitary with resultant progesterone secretion from the luteinized structures. At 25 rat units, the uterine mucosa showed less transformation, and at the lower doses no such change occurred.

Beef pituitary interstitial cell stimulating hormone (ICSH). Since administration of an ICSH-like urinary hormone (chorionic gonadotrophin) repaired what appeared to be a defect in gonadotrophic secretion resulting from the hypothalamic lesion, it was decided to attempt restoration with pituitary ICSH. Purified beef ICSH prepared by salt fractionation and having a minimal effective dose of 0.025 mg was given to 2 rats which were obese and showed constant vaginal cornification. A total dose of 1 mg of ICSH was given intraperitoneally in 4 daily doses. Twenty-four hours after first injection the vaginal smear contained leucocytes and by 48 hours the smear was typical of diestrus and remained so. On the fifth day the ovaries were examined grossly and histologically and showed a single set of newly formed corpora lutea and the interstitial tissue was indistinguishable from that of normal controls. Distended oviducts were not observed and ova were not found in sections of oviducts. Breeding behavior was not investigated.

Discussion. The demonstrations by Dey *et al.*(1) and by Hillarp(3) that destructive lesions in the hypothalamus may result in failure of corpus luteum formation have been confirmed in this study. Replacement therapy with luteinizing hormone (chorionic gonadotrophin or pituitary ICSH) resulted in the formation of functional corpora lutea. A similar result was obtained in the guinea pig by Brookhart, Dey and Ranson(2). Whether the location of lesions represents the actual center of control of luteinization or a point along the pathways from the center, as suggested by Hillarp, has not been investigated.

The changes seen in the pituitaries of the rats with prolonged cornification were suggestive of the changes found after prolonged estrinization from exogenous source(14) *i.e.*, disappearance of gonadotrophic basophils and profound degranulation of the acidophils. However, the animals with hypothalamic lesions, although showing effects of chronic estrinization, appeared to be continuously forming new follicles, indicating that secretion of follicle stimulating hormone must then have been occurring to some extent in spite of the decrease in gonadotrophic basophils. The location of the hypothalamic lesions could not be correlated with the variability in adiposity and ovarian function.

Summary and conclusions. 1. Bilateral hypothalamic lesions ventral to the ventromedial nucleus in female rats resulted in persistent vaginal cornification in the majority of animals. Thyroid morphology and I^{131} uptake in obese rats with constant cornification indicated increased thyroid activity. The ovaries of those showing constant cornification contained follicles of all sizes but no corpora lutea. The interstitial tissue was not atrophic. 2. The follicles were readily converted into corpora lutea by administration of luteinizing substances, both human chorionic gonadotrophin and pituitary interstitial cell stimulating hormone. The interpretation is made that secretion of luteinizing factor by the pituitary may be impaired by small, bilateral hypothalamic lesions ventral to the ventro-medial nucleus.

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Susceptibility of Hamsters to Peripherally Inoculated Japanese B and St. Louis Viruses Following Cortisone, X-Ray, Trauma.* (23102)

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In making cross immunization studies with certain of the better known Group B encephalitis viruses, Hammon and Sather(1) employed the Syrian hamster. These animals were effectively challenged by a peripheral route with West Nile (WN) virus and with Murray Valley (MVE) encephalitis viruses, since both of these agents produced fatal infections through high virus dilutions when inoculated either subcutaneously or intraperitoneally. In these experiments previous immunization had been carried out through inapparent infection with St. Louis (SLE) and Japanese B (JBE) viruses. Similar challenge could not be made, however, in the other direction using JBE or SLE viruses after immunization with WN, MVE or other Group B viruses. An adequately susceptible experimental animal for peripheral challenge with JBE and SLE viruses was much desired, but none was currently available. Among the possible uses for an animal highly susceptible to quantitative peripheral challenge were: (1) a more suitable potency test for specific formalin inactivated virus vaccines; (2) a means for testing for immunity induced by previous infection with an immunologically related virus. Our most immediate interest in finding such an animal was to test for immunity against JBE and SLE viruses following experimental infection by a related avirulent living virus immunizing infection to be supplemented by one or more injections of specific formalin inactivated virus vaccine as discussed by Hammon and Sather(1) and Hammon, Sather, Lennette and Reeves(2). Use of an avirulent WN virus infection had been specifically suggested to precede the use of a JBE inactivated virus vaccine, since use of the latter alone had not appeared to give adequate protection in adult, military usage (3). Flexner and Amoss(4) reported that "normal monkey or horse serum, isotonic salt solution, and Ringer's and Locke's solution when injected into the meninges promote infection with the virus of poliomyelitis introduced into the blood, the nose, or the subcutaneous tissues." Zwick, Seifried and Witte (5) found that cutaneous inoculation of rabbits with Borna virus succeeds in producing disease in exceptional instances only, but that disease is easily brought about by such inoculation if an injury is produced at the same time in the central nervous system by injection of non-specific fluids (normal rabbit serum, normal horse serum, or isotonic chloride solution). Working with mice, Sawyer and Lloyd(6) showed that intraperitoneal inoculation of yellow fever virus, which was without pathological effect in the intact adult animal, produced encephalitis if the brain was injured. They used 2% starch in 0.9% NaCl solution, and inoculated the animal with 0.03 cc intracerebrally. Simple trauma to the brain with a needle also had a provocative effect. Later, Webster and Clow(7) indicated a similar facilitating effect of brain injury with the virus of SLE. Burnet and Lush(8) found that brain injury enhanced the effect of louping ill virus. On the other hand, Olitsky, Cox and Syverton(9) were unable to induce disease with vesicular stomatitis virus injected intraperitoneally even though the brain was simultaneously traumatized. In Eastern equine encephalitis virus infection, King(10) reported that traumatization of the brain by simple needling had insignificant effect. However, he found that a non-specific irritation by injection of glycerin into the peritoneal cavity increased incidence of dis-

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† From thesis submitted as partial requirement for Doctor of Public Health Degree. Present address: Serum and Virus Laboratories, A. Gouza, Cairo, Egypt.

ease in mice to subcutaneous inoculation of this virus. Marie(11) reported a similar action with rabies virus after intraperitoneal injection of India ink. It has been demonstrated that cortisone produces a highly significant enhancement of experimental intracerebral infection with the Lansing strain of poliovirus in the hamster(12,13). Adult mice can be lethally infected with Coxsackie virus if cortisone is administered in a single dose prior to the inoculation of the virus(14). Susceptibility of such adult mice is reported to be fully as great as that of infant mice. The only work with cortisone which we have found reported to date using any of the Group B encephalitis viruses is that by Volmer and Hurlbut(15) with JBE virus. They attempted to use cortisone as a therapeutic agent in mice. Instead of reducing mortality of mice inoculated subcutaneously, they found that this product increased it. The effect, however, was not sufficiently marked to encourage us to employ this animal in our work, at least not until hamsters had been tried. It would appear from the studies of a number of investigators(16-22) that cortisone most probably acts through inhibition of assimilation of antigen and of the synthesis of antibody globulin. Evidence of the effect of X-irradiation on immunity is found in animals irradiated shortly before immunization with non-living antigens. The effects produced by X-ray on innate and acquired immunity of the intact animal have been reviewed by Taliaferro(23). More recent reports(24-27) have shown that prior exposure of the whole animal to X-ray increases its susceptibility to selected viral agents. This effect may be due to an impairment of the antibody response. Syverton, *et al.*(24) showed that when both X-ray and cortisone were employed a markedly synergistic effect could be demonstrated. Adult mice normally not susceptible to poliovirus, Coxsackie virus, and certain other agents developed rapidly progressive lethal infection following this combination treatment.

Materials and methods. Syrian hamsters obtained from the Lakeview Hamster Colony, were used in all the experiments here reported. They were about 8 weeks old, of both sexes. A frozen pool of JBE Nakayama strain virus, mouse passage 45, hamster passage 3, with an LD_{50} of $10^{-7.5}$ in mice was used throughout. St. Louis Webster strain of virus as a frozen mouse brain suspension with an infecting titer in mice of $10^{-7.8}$ was employed when this agent was used. The susceptibility of each hamster to either of these viruses was tested by inoculating it subcutaneously with 0.1 cc of a suitable dilution of virus. The cortisone used was manufactured by Sharp and Dohme. Before starting an experiment with cortisone, each hamster was weighed and the hamsters were put in groups having as nearly as possible the same weight. It was usually possible to use only one sex of animals in any one experiment. However, from experiment to experiment, no sex differences in susceptibility were ever detected. The total dose of cortisone in milligrams was calculated on the basis of body weight of the hamster in grams. The total dose of cortisone was given in 4 daily injections. On the first day of the experiment, the hamster received its first dose of cortisone. On the second day, following the cortisone, virus was injected by the subcutaneous route. Approximately 35% of the total cortisone dosage was given the first day, 25% on the second, and 20% on each of the last 2 days. Roentgen rays‡ were generated by a 4 milliampere current operating at 100 kilovolts and were filtered through 1.0 mm of aluminum (HVL 1.1 mm AL). The size of the field was 20 cm square; individual readings taken at selected points in the target area with a Victoreen r meter varied from 4.65 to 4.90 roentgens in air per minute. A single massive dose of radiation was administered to the whole body of the animal at a skin target distance of 78 cm at an average rate of 4.7 r per minute. The hamsters were put in a wire basket in a manner which allowed them to sleep side by side without too much movement. The basket was covered with a used X-ray film which was fastened to the basket by adhesive tape.

‡ The work with X-rays was done with the advice, assistance and equipment of Dr. F. S. Cheever, a member of this Department.

TABLE I. Effect of Cortisone on Susceptibility of Hamsters to Subcutaneous Inoculation of JBE Virus.

Test	Cortisone,* mg	Virus inoc.†	Mortality ratio	Mortality %
I	.05	Virus	5/15	33.3
	"	None	0/15	.0
	None	Virus	1/15	6.6
II	.1	Virus	5/ 6	83.0
	"	None	0/ 6	.0
	None	Virus	1/ 6	16.6
III	.2	Virus	10/10	100.0
	"	None	0/10	.0
	None	Virus	0/10	.0
IV	.3	Virus	15/15	100.0
	"	None	1/15	6.6

* Cortisone by g body wt.
† 0.1 cc of 10^{-1} mouse brain suspension.

Results. Exp. 1 and 2. Brain irritation and intraperitoneal glycerin with JBE virus. Cerebral irritation with 2% starch and peritoneal trauma with 0.5% glycerin following subcutaneous inoculation of a 10^{-1} dilution of JBE virus were both tried. Mortality changed from 10% in the controls to only 30% and 40% respectively. Since this was not an adequate mortality for our purposes this line of investigation was not followed further.

Exp. 3 and 4. Various dosages of cortisone and a 10^{-1} dilution of JBE. In Exp. 3 four tests were performed using different total doses of cortisone. In the first a total dose of 0.05 mg/g body weight was employed; in the second 0.1 mg; in the third 0.2 mg; in the fourth 0.3 mg. In each test, with one exception, there were 3 basic groups of hamsters: (1) treated with cortisone and injected with the virus; (2) treated with cortisone only; and (3) injected with virus only. In the fourth test, Group 3, the virus controls were omitted. Hamsters in all tests were watched for a period of 3 weeks. Those which died or were moribund during this period were autopsied and the liver, spleen, and blood were cultured on blood agar. Deaths caused by excess cortisone could be detected by this means, since cultures from organs of these animals showed heavy bacterial growth. It can be seen from Table I that the dose of 0.05 mg of cortisone/g body weight plus virus gave 33% mortality; the dose of 0.1 mg plus virus gave 83% mortality; and the doses of 0.2 and 0.3 mg gave 100% mortality. No deaths occurred in the cortisone control groups except in the last test, in which the 0.3 mg dosage was used. Pathogenic bacteria were found only in certain of the autopsied hamsters of this last group, those receiving the greatest amount of cortisone. Only 6.6 to 16.6% of the hamsters died in the virus control groups. From these results it appeared that a total dose of 0.2 mg cortisone/g body weight was the most promising one. Therefore, a confirming experiment (No. 4) was performed, using this dose of cortisone and virus again. Eight hamsters were used in each group. Results of this experiment confirmed the findings of the previous one. There was 100% mortality in those receiving cortisone and virus, 12.5% in those receiving only virus, and 0% in those receiving only cortisone.

Exp. 5. Titration of JBE in cortisone-treated hamsters. An experiment was then performed in the manner of a virus titration. A 10-fold serial dilution of virus was inoculated subcutaneously in groups of hamsters, each treated with a total dose of 0.2 mg cortisone/g body weight in the way described above. Five hamsters were used for each virus dilution. Results of this titration are shown in Table II. The LD_{50} titer of JBE virus as determined by subcutaneous inoculation was greater than 10^{-6}, while in normal hamsters it is regularly less than 10^{-1}.

Exp. 6. Cortisone and a 10^{-1} dilution of SLE. In this experiment hamsters were treated with cortisone in exactly the same manner as in the JBE experiments using a total of 0.2 mg/g body weight. On the second day of the experiment the hamsters were inoculated subcutaneously with 0.1 cc of a 10^{-1} dilution of SLE virus. Results are shown in Table III. Cortisone increased the mortality of hamsters to subcutaneous inocu-

TABLE II. Titration of JBE Virus Subcut. in Hamsters Treated with Cortisone.

	Virus dilution and mortality ratio					
Cortisone*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
.2 mg	5/5	5/5	4/5	5/5	4/5	4/5
None	0/5	0/5	0/5	——not done——		

* Cortisone by g body wt.

TABLE III. Effect of Cortisone on Susceptibility of Hamsters to Subcutaneous Inoculation of SLE Virus.

Cortisone,* mg	Virus inoc.†	Days of death after virus inoc.	Mortality ratio	Mortality %
.2	Virus	7, 9, 11, 14	4/5	80
"	None	‡	0/5	0
None	Virus	‡	0/5	0

* Cortisone by g body wt.
† .1 cc of 10^{-1} mouse brain suspension.
‡ No deaths.

lation of a 10^{-1} dilution of SLE virus from 0% to 80%.

Exp. 7. Titration of SLE in cortisone-treated hamsters. In this experiment the hamsters were divided into 2 groups: (1) 40 hamsters treated with 0.2 mg cortisone/g body weight and inoculated with serial 10-fold dilutions of SLE virus (5 with each dilution) as shown in Table IV; (2) 15 hamsters inoculated (5 each) with 10^{-1} through 10^{-3} dilutions of the virus only. The mortality of those hamsters given cortisone with different dilutions of the virus was fairly constant at 3 or 4 out of 5 through the 10^{-7} dilution, but was never 100%. Because of this an LD_{50} cannot be calculated. No hamsters died in the non-cortisone treated groups. In this experiment no cortisone control group was used; however, all moribund hamsters were autopsied and cultures made without detecting any evidence of death due to bacterial infection from excessive cortisone.

Exp. 8 and 9. X-ray with a 10^{-1} dilution of JBE. In 2 essentially identical experiments, test hamsters each received a dose of 450 r of X-ray on the first day, and on the second day each was given 0.1 cc of a 10^{-1} dilution of virus subcutaneously. Groups of virus control and X-ray control hamsters were included in each of the experiments. In the first, 15 hamsters were used in each group, in the second 8 were used. In Experiments 8 and 9, 450 r of X-ray increased susceptibility of hamsters to subcutaneous inoculation of JBE virus (Table V). A mortality of about 85% was observed in the test groups, and of only 6.6% to 12.5% in the X-ray control groups.

TABLE IV. Titration of SLE Virus Subcut. in Hamsters Treated with Cortisone.

	Virus dilution and mortality ratio							
Cortisone*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
.2 mg	3/5	4/5	3/5	4/5	3/5	4/5	4/5	2/5
None	0/5	0/5	0/5	———	not done	———		

* Cortisone by g body wt.

TABLE V. Effect of 450 r X-ray on Susceptibility of Hamsters to Subcutaneous Inoculation of JBE Virus.

Exp.	Treatment	Virus inoc.*	Mortality Ratio	Mortality %
8	450 r	Virus	12/14	85.7
	"	None	1/15	6.6
	None	Virus	1/15	6.6
9	450 r	Virus	7/ 8	87.5
	"	None	1/ 8	12.5
	None	Virus	1/ 8	12.5

* .1 cc of 10^{-1} mouse brain suspension.

Exp. 10. X-ray and cortisone with a 10^{-1} dilution of JBE. X-ray was administered as described above, but in doses of 150, 300 and 400 r. Cortisone was administered in doses of 0.10, 0.15 and 0.20 mg/g body weight. Various combinations of cortisone and X-ray were tried, hoping to find a more effective method of increasing susceptibility of hamsters to subcutaneous inoculation of JBE virus.

Seven groups of hamsters were used in this experiment (Table VI). Each of the first 4 groups was composed of 16 animals and each of the fifth and sixth groups was composed of 14 animals. Each of these groups was divided into 2 subgroups; one subgroup received X-ray and cortisone plus virus; the other subgroup acted as a control for the X-ray and cortisone without virus. The seventh group, of 8 hamsters, was inoculated with the virus only and acted as a virus control group. The total dose of X-ray was given on the first day of the experiment, and cortisone was given over a period of 4 days beginning on the day X-ray was administered. Virus in a 10^{-1} dilution was given on the second day of the experiment. The various combinations of X-ray and cortisone used gave a 100% mortality rate in all the test groups which received virus (Table VI). Also, there were deaths in the control treatment groups except in that receiving the smallest dose of both X-ray and cortisone. Deaths in the virus control group

TABLE VI. Effect of Cortisone and X-ray on Susceptibility of Hamsters to Subcutaneous Inoculation of JBE Virus.

	Cortisone in mg/g body wt								
	.1			.15			.2		
X-ray in r units	Virus inoc.	Mortality Ratio	%	Virus inoc.	Mortality Ratio	%	Virus inoc.	Mortality Ratio	%
150	0	0/8	0	0	1/8	12.5	0	1/8	12.5
	+	8/8	100	+	8/8	100	+	8/8	100
300	0	1/8	12.5	0	1/7	14.2			
	+	8/8	100	+	7/7	100			
450	0	2/7	28.5						
	+	7/7	100						

Mortality ratio in 8 hamsters inoculated with the virus only was 1/8 or 12.5%.

were 12.5%. It can be concluded that a combination of 150 r X-ray plus a total dose of cortisone of 0.1 mg/g body weight did not produce deaths due to over-dosage but did increase the susceptibility of the animals to death as a result of peripheral inoculation of virus. Larger doses led to deaths in the controls.

Exp. 11. Titration of JBE in X-ray plus cortisone-treated hamsters. In this experiment serial 10-fold dilutions of virus were inoculated subcutaneously in groups of 5 hamsters treated with 150 r of X-ray plus 0.15 mg cortisone/g body weight, a dosage decided upon following Exp. 10. For comparison of the effect of 0.2 mg of cortisone alone, shown to be non-toxic but effective in previous experiments, similar groups of virus-injected hamsters were treated in this manner. Virus controls were also included.

The LD_{50} titer of JBE virus in the subcutaneously inoculated hamsters given X-ray and cortisone was between 10^{-4} and 10^{-5} (Table VII). Cortisone in a 0.2 mg dose without X-ray, however, gave an LD_{50} of $>10^{-6}$ in the virus titration.

Discussion. Hamsters, with few exceptions, do not become sick or die from subcutaneous inoculation of JBE or SLE virus(28). From the work of previous investigators it was found that brain irritation or peritoneal irritation may enhance the susceptibility of some animals to peripheral inoculation of certain viruses. Since we were interested in finding an animal susceptible to subcutaneous inoculation of these viruses, these 2 methods were tried on hamsters, but without too much success. Since the adult mouse is slightly more susceptible to peripheral injection of JBE virus than the hamster treated by either of these 2 methods, this did not appear to be worthy of further testing for our purposes. The possibility was considered that the poor effect of brain irritation and intraperitoneal glycerin on susceptibility of hamsters to subcutaneous inoculation of JBE virus might be due to the fact that neither of these 2 methods had an inhibitory effect on development of specific neutralizing antibodies which might be playing an important role in protection of the hamsters. Other methods which might have such an effect were selected for subsequent experiments.

TABLE VII. Titration of JBE Virus Subcut. in Hamsters Treated with Cortisone Alone or with Combination of Cortisone and X-ray.

Treatment	Virus dilution and mortality ratio					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
.2 mg cortisone	5/5	5/5	4/5	5/5	4/5	4/5
150 r X-ray + .15 mg cortisone	5/5	5/5	5/5	5/5	0/5	2/5
None	0/5	0/5	0/5	—not done—		

Whether or not the results were due to immunologic factors, it is obvious that a suitably administered regime of cortisone greatly enhanced susceptibility of the hamsters to peripheral injection of these viruses. The susceptibility to JBE, in fact, practically equalled that which occurs when the virus is given intracerebrally. By maximum irradiation with X-rays compatible with survival of most of the controls the susceptibility of hamsters to subcutaneous inoculation of JBE virus increased from 6.6% or 12.6% in non-irradiated hamsters to about 85%. Further

alteration of time and dosage factors might have improved this.

Treatment of hamsters with X-ray plus cortisone in the maximum combination dose tolerated rendered hamsters more highly susceptible to JBE virus than maximum doses of X-ray alone. This effect might be due to a synergistic action between X-ray and cortisone as suggested by Syverton, *et al.*(24). However, since a simultaneous comparison of the effect of cortisone alone with the best combination treatment tested showed the former to be superior to the combination, and since the use of cortisone alone was a simpler procedure to employ and beset with fewer variables, the combination treatment was not investigated further. Subsequent work on immunologic response of the inoculated animals, with and without cortisone, and on evaluation of immunization procedures reported in subsequent papers was carried out using cortisone alone.

Summary. 1) Brain irritation and intraperitoneal glycerin were tried to enhance susceptibility of hamsters to subcutaneous inoculation of JBE virus. Both of these methods have a slight enhansive effect, but not of the degree desired for the purposes of this study. 2) A total dose of 0.2 mg cortisone/g body weight, when given to hamsters as described, rendered them highly susceptible to subcutaneous inoculation of JBE virus and to only a slightly less degree to SLE virus. 3) It was found that a dose of 450 r of X-ray detectably increased susceptibility of hamsters to subcutaneous inoculation of JBE virus. This was the maximum dose of X-ray tolerated, and increase in susceptibility observed was less marked than with cortisone. 4) Combined treatment of hamsters with the maximum tolerated combined doses of X-ray and cortisone (150 r of X-ray and 0.15 mg cortisone) rendered them much more highly susceptible than X-ray alone; however, the combination tested was not as effective as cortisone alone in a subtoxic dose. Cortisone at a dosage of 0.2 mg/g body weight was adopted as a standard procedure for subsequent work. The increase in susceptibility to JBE virus was in excess of 100,000 fold, rendering the animals almost as susceptible to peripheral inoculation as to intracerebral.

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Immunologic Response and Pathogenesis of Japanese B Infection In Peripherally Inoculated Normal and Cortisone Treated Hamsters.* (23103)

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Most Syrian hamsters are not susceptible to clinical disease when inoculated subcutaneously with Japanese B encephalitis virus (JBE)(1). In our hands the mortality rate following a subcutaneous injection of a 10^{-1} dilution of mouse brain suspension is usually 10% and rarely do any animals developing clinically detectable signs ever survive. Since the immune mechanism is well established as a principal factor in the defense against certain invading viruses(2,3), it seemed logical to consider the relation it might bear to the low susceptibility to JBE virus exhibited by hamsters when inoculated by the subcutaneous route. It was conceivable that early formation of antibody might be responsible for arresting infection prior to development of observable signs of disease. It was found that cortisone markedly increased susceptibility of hamsters to subcutaneous inoculation of JBE virus(4). As the mode of action of cortisone is most probably through inhibition of antibody production, this lent support to the hypothesis. Investigations were undertaken to determine rate of antibody formation and virus multiplication in the brain and blood in normal and in cortisone treated hamsters inoculated with the virus subcutaneously. It was expected that these experiments might shed some light on the pathogenesis of the disease as well as on the protective mechanism.

Materials. A frozen suspension of the JBE Nakayama strain of virus in mouse passage 45 was used throughout. This had an LD_{50} of 10^{-7} when titrated intracerebrally in mice. Syrian hamsters obtained from the Lakeview Hamster Farm and about 8 to 10 weeks old, and randomly selected from either sex were used. White mice of the CFW strain, 3 to 4 weeks old, were employed for all virus titrations and neutralization tests. *Observations on JBE virus infection following subcutaneous inoculation of virus in normal hamsters. Exp. 1.* As an orienting preliminary experiment, 24 hamsters were inoculated subcutaneously with 0.1 cc of a 10^{-1} dilution of virus. On the 1st, 2nd, 4th, 6th, 8th, 11th and 21st days after inoculation 3 hamsters were picked at random among those not appearing ill. They were exsanguinated under anaesthesia and the sera from each day's group were pooled. Brains were also removed and pooled. On each of these days, a portion of the pooled sera and of the pooled brain suspensions was tested quantitatively for virus. Other portions of the serum pools of all hamsters from the 4th day on were tested at one time for presence of neutralizing antibodies by the intracerebral mouse test, using the serial virus dilution method(5). In this experiment 3 hamsters were found sick on the 7th day after inoculation. They were sacrificed at that time, their brains were pooled and tested quantitatively for virus just as in the case of those which were apparently normal. No others were observed to be ill at any time.

Results. JBE virus was present in the sera of hamsters bled on 1st and 2nd day after inoculation and disappeared from the sera by the 4th day (Table I). Beginning on 4th day after inoculation virus was observed in brains of those animals with no signs of illness, reaching its maximum titer of 10^{-4} on 6th to 8th day and decreasing by the 11th day. No virus was found on 21st day. By contrast, brains of those observed to be sick and sacrificed on 7th day showed a titer of $10^{-5.7}$. Results of neutralization tests to JBE virus on the hamster sera are also shown in Table

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TABLE I. Normal Hamsters: Log Titer of Neutralizing Antibodies* and of JBE Virus in Pools of Sera, and of Virus in Pools of Brains at Intervals after Subcutaneous Inoculation of Virus.

Material	Tested for	Interval in days: 1	2	4	6	7	8	11	21
Serum	Virus	3.0	1.8	neg†	neg	———	———	§———	
Brain	"	neg	neg	2.5	4.1	5.7‡	4.1	1.5	neg
Serum	N.A.*	———§———		.6	.9	§	1.0	1.0	1.7

* Tests for N.A. (neutralizing antibodies) made by intracer. method, expressed as log neutralization index. † neg = no virus detected—brains in 20% suspension and blood sera undiluted. ‡ Sick hamsters. § Not tested.

I. Neutralizing antibodies to JBE of a titer usually accepted as significant in clinical diagnostic work with this type of test (neutralization index 50 or 1.7 logs)(5) did not appear until possibly on the 21st day. This is partly explained by the fact that the virus from the ampoule used in this neutralization test was found to have a titer which had fallen since previous titrations of other ampoules. Its titer in the control normal rabbit serum was only 10^{-6}. Since this was but an orienting experiment, the neutralization tests were not repeated.

Exp. 2. Another experiment was performed using a larger number of hamsters. Sixty animals were inoculated subcutaneously each with 0.1 cc of a 10^{-1} dilution of JBE virus. Three were sacrificed at each of several different intervals after time of injection, starting at 6 hours, then 12 hours and 24 hours, then daily up to the 13th day, and finally weekly up to the 42nd day. The hamsters sacrificed were dealt with as in the 1st experiment except that tests for virus on serum and brains were made separately rather than by pools. The sera which did not contain detectable virus were tested for presence and titer of neutralizing antibodies to JBE virus, for the most part in pools by interval day, but all tests were made at one time. The sera from the 4th through the 8th days were also titrated using the method of intraperitoneal inoculation of suckling mice which is believed by many to be more sensitive for detecting small amounts of antibody(6).

Results. Viremia was already present 6 hours after subcutaneous inoculation of virus, reached its maximum at about 24 hours and had disappeared by the 3rd or 4th day (Table II). Virus appeared in the brain on the 4th day, reached its maximum ($10^{-2.7}$ to $10^{-3.5}$) around the 7th or 8th day and disappeared after the 10th or 12th day. In this experiment 2 hamsters were found sick and paralyzed on the 6th day after inoculation. The titer of the virus in their brains (not shown in table) was $10^{-5.6}$ for one and $10^{-5.4}$ for the other, higher than the levels found in any of those observed not to be ill. Neutralizing antibodies as detected by the intracerebral test were at a "significant," detectable level by the 5th day after inoculation, fluctuated during the next week, and then rose rather steadily to reach a maximum titer around the 27th or 34th day after inoculation (Table II). Intraperitoneal type neutralization tests performed on the sera of the 4th through the 8th day, also shown in the table, confirmed the very early appearance of serum antibodies; a neutralization index of 1,000 in the sera of the 4th and 5th days, and greater than 10,000 in sera of the 6th, 7th and 8th days.

Observations on JBE virus infection following subcutaneous inoculation in cortisone treated hamsters. An experiment was performed on the same lines but using hamsters treated with cortisone in a dose of 0.2 mg/g body weight given over a period of 4 days(4). Each of the 40 hamsters used in this experiment was inoculated with 0.1 cc of a 10^{-1} dilution of JBE virus subcutaneously 1 day after the 1st cortisone injection. Three animals were sacrificed daily regardless of their clinical status. During the last few days all hamsters were ill.

Results. Virus was observed in the sera of hamsters beginning on the 1st day after inoculation, reached its maximum about the 3rd day and disappeared from the sera after the 4th

TABLE II. Normal Hamsters: Log Titer of Neutralizing Antibodies* and of JBE Virus in Sera, and of Virus in Brains at Intervals after Subcut. Inoculation of Virus.

Material	Test for	Hamster No.	Interval in hr: 6	12	24	Interval in days: 2	3	4	5	6	7	8	9	10	11	12	13	20	27	34	41
Serum	Virus	1		1.6	2.0	1.2	neg†	neg	neg	—	—	—	—	—	—	‡	—	—	—	—	—
		2		.5§	1.2	2.5	1.2	.3	"							"					
		3			‡	2.9	1.0	neg	"							"					
Brain	"	1	neg	neg	neg	neg	neg	2.4	2.4	1.5	2.5	2.7	1.8	2.6	neg	neg	—	—	‡	—	—
		2	"	"	"	"	"	1.5	1.6	1.4	3.1	.7	1.7	1.4	"	.7			"		
		3	"	"	"	"	"	1.1	2.5	1.4	2.5	3.5	1.6	2.8	"	neg			"		
Serum	N.A.*	i.c.‖ 1,(2,3)¶	—	—	‡	—	.6	1.0	1.8	2.2	2.5	2.2	1.8	2.3	1.0	1.2	2.0	2.3	1.6	3.0	2.6
		i.c. 2 ¶	—	—	—	—	—	—	‡	—	—	—	—	—	—	—	1.6	2.5	2.7	‡	—
		i.c. 3 ¶	—	—	—	—	—	—	"	—	—	—	—	—	—	—	1.9	1.7	3.4	"	
		i.p.** 1,2,3§	—	—	‡	—	—	3.0	3.0	>4.0	>4.0	>4.0	—	—	—	—	‡	—	—	—	—

* Tests for N.A. (neutralizing antibodies) made by intracer. method and expressed as log neutralization index.
brains in 20% suspension and blood sera undiluted.
neutralization test.
neutralization test.
† neg = no virus detected—
‡ Not done.
§ Pool of sera from hamsters 1, 2 & 3.
‖ i.c. = intracerebral mouse
¶ Pool of 3 sera, except sera on 13th, 20th and 27th days, which were individual sera.
** i.p. = intraper. infant mouse

day (Table III). Virus was detected in the brains of the hamsters beginning on the 3rd to the 5th day after inoculation, reached its maximum ($10^{-6.8}$ to $10^{-7.2}$) on the 7th or 8th day, and remained at a high level (in most instances above 10^{-5}) to the end of the experiment when all animals were moribund or dead. Also found in Table III are the results of neutralization tests on the sera not found to contain virus. Neutralizing antibodies attained a "significant" level (neutralization index of 50 or 1.7 logs) in only 2 animals, one on the 7th and one on the 11th day.

Discussion. Comparison of the results obtained with cortisone and of those obtained from the experiment with normal hamsters showed the following:

(a) In cortisone treated hamsters viremia persisted for a longer time, for instead of disappearing after the 2nd or 3rd day after inoculation, as in normal animals, it did not disappear until after the 4th day in those treated with cortisone. Furthermore, the titer of virus detected in the blood continued to rise after it had essentially disappeared in normal hamsters and attained at that time a higher titer than that of the earlier peak in normal animals.

(b) Virus was observed in the brains of cortisone treated hamsters slightly earlier (on the 3rd day instead of the 4th) reached its maximum on about the 7th day, behaving essentially like that of the few normal hamsters which became ill, and continued to be on a high level of about 10^{-5} or 10^{-6} to the end of the experiment. These levels were much higher than those attained in most of the hamsters not given cortisone. Virus levels in the brains of normal hamsters were somewhat higher, however, on the 4th and 5th days than in those of the cortisone treated animals. No explanation for this is obvious but it is quite possibly due to individual variation and of no significance.

(c) Neutralizing antibodies appeared considerably later and generally did not attain "significant" levels in cortisone treated hamsters. The antibody response of those not given cortisone was of a higher order, as had been anticipated.

TABLE III. Cortisone Treated Hamsters: Log Titer of Neutralizing Antibodies and of JBE Virus in Sera, and of Virus in Brains at Intervals after Subcutaneous Inoculation of Virus.

Material	Test for	Hamster No.	Interval in days 1	2	3	4	5	6	7	8	9	10	11
Serum	Virus	1	1.6	1.3	3.0	1.0	neg	neg					
		2	1.2	1.0	1.5	1.5	"	"					
		3	1.3	1.0	4.9	3.0	"	"					
		Mean*	1.4	1.1	3.1	1.7	"	"					
Brain	"	1	neg	neg	neg	neg	.3	2.0	7.2	5.5	5.0	6.1	6.0
		2	"	"	.3	"	.5	.7	6.5	6.8	6.1	5.2	5.5
		3	"	"	1.3	1.9	.5	1.3	7.0	5.6	6.3	5.1	4.8
		Mean	"	"	.5	.6	.4	1.3	6.9	6.0	5.8	5.4	5.4
Serum	N.A.	1					.7	.0	.4	1.4		.5	1.7
		2					.7	.5	1.5	1.0	1.0	1.0	1.3
		3					.3	.7	2.0		1.3	1.3	1.2
		Mean					.6	.4	1.3	1.2	1.2	.9	1.4

* Geometric mean.
See Tables I and II for interpretation of other symbols and abbreviations.

Hodes and Webster(7) observed that mice inoculated subcutaneously with the virus of SLE failed to develop clinical evidence of encephalitis, but quickly developed immunity against subsequent intracerebral inoculation. They claimed that this immunity, however, was not accompanied by development of virus neutralizing antibodies in the peripheral blood. Such antibodies were noted some 8 weeks after subcutaneous inoculation, at which time, however, immunity to intracerebral inoculation was lost.

Brown, *et al.*(2) showed that humoral antibodies against the virus of SLE can be demonstrated in the sera of hamsters inoculated by the subcutaneous, intracerebral, intravenous and oral routes provided the animal survived the infection 7 days or more. They showed that after subcutaneous inoculation of SLE virus in hamsters, antibodies might appear in the serum within 48 hours after inoculation. They persisted at a high titer for many weeks. Our work tends to confirm this rather than that reported in mice by Hodes and Webster, although viremia was still present in our animals at 48 hours so no test was made for neutralizing antibodies.

From the results of our experiments on normal hamsters inoculated subcutaneously with JBE virus, it appears most probable that the early appearance of antibodies is the main factor that renders hamsters insusceptible to disease from subcutaneous inoculation. This early production of antibodies probably prevented free virus multiplication in the hamster brains and kept the virus at a low level (below 10^{-4}), a level which does not lead to apparent disease. Only in a very low percentage of hamsters, which possibly failed to develop early antibodies, did the virus multiply freely in their brains, reach a titer of about 10^{-5} and lead to obvious illness and death.

Through the action of cortisone which most probably is by inhibition of antibody production, susceptibility of hamsters to subcutaneous inoculation of JBE virus was increased. It appears that cortisone delayed and inhibited antibody production so that the virus multiplied freely and remained for a longer time in the blood, and appeared earlier and reached a higher titer in the brain.

It is interesting to note that this virus infection of hamsters which has been studied following subcutaneous injection and appears to cause clinical illness and death by damage to the central nervous system, is associated with viremia and that the eventual course—clinical illness and death—does not appear to depend upon the breakdown of any supposed "blood-brain" barrier. As in the human host, by natural infection, most normal hamsters are infected and respond immunologically but without disease, while a few become ill. In the hamster virus appears very early in the blood, possibly multiplying there or elsewhere

and released into the blood. Then with apparent absolute regularity it is found in the brain, probably multiplying there. However, this involvement of the brain generally remains at a subclinical level and after what may very likely be a lively combat between virus multiplication and antibodies, with serum antibody levels and brain virus levels fluctuating considerably as a result, the infection with brain involvement is usually overcome. Obviously antibody does not prevent virus from penetrating a "blood-brain barrier" in the normal animal for the virus regularly appears in the brain. This is not a reflection of what is in the blood, for by the time it appears in the brain it is no longer detectable in the blood. Nevertheless, antibody may play a very important role in controlling the extent of virus invasion in the central nervous system provided it is available sufficiently soon. Cortisone, as used, probably effects an adequate delay in this response to change the outcome so that essentially 100% of the animals become ill and die.

Summary. Most hamsters are not susceptible to disease following subcutaneous inoculation of JBE virus, probably due to early production of antibodies. Normal hamsters when inoculated with JBE virus subcutaneously, developed viremia 6 hours after inoculation. This viremia reached its maximum after 24 hours and disappeared by the 4th day. Virus appeared in the brain on the 4th day and reached its maximum around the 7th or 8th day and disappeared after the 10th or 12th day. Neutralizing antibodies to JBE virus were detected in their sera beginning on the 4th or 5th day and reached their maximum titer around the 27th or 34th day after inoculation. In cortisone treated hamsters viremia reached its maximum by the 3rd day and disappeared after the 4th day. Virus was detected in the brains of these hamsters from the 3rd day after inoculation, reached its maximum on the 7th or 8th day and remained at a high level to the end of the experiment. Neutralizing antibodies to JBE virus attained a significant level (1.7 logs) in only 2 animals and that on the 7th and the 11th days. It seems probable that cortisone, through partial inhibition of antibody production, allowed the virus to multiply freely in the brain and so rendered hamsters highly susceptible to subcutaneous inoculation of the JBE virus.

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Attempts to Immunize Hamsters to West Nile Virus; Passive, Passive-Active and Active Methods.* (23104)

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As outlined previously(1,2) we have planned to test in laboratory animals the effectiveness of immunization against Japanese B (JBE), St. Louis (SLE), and Murray Valley (MVE) encephalitis viruses by primary immunization with living West Nile (WN) virus supplemented with an inactivated virus vaccine of one of the other types. The hamster proved to be a suitable host for test challenge by a peripheral route following immunizing procedures, thus simulating naturally acquired infection from a mosquito bite (2). Susceptibility of the hamster to MVE and WN was adequate without any modifying procedures(1,3), but to obtain adequate susceptibility for JBE and SLE challenges it was necessary to treat the hamsters with cortisone(2). Thus, an animal species other than man was available for suitable quantitative challenge after immunizing procedures. Monkeys, mice and other laboratory animals were not suitable.

The next problem was a method of producing the desired basic immunization with living WN virus, since hamsters are extremely susceptible to peripheral injection of this virus. High dilutions of WN virus were first employed to determine whether a dosage could be found which would produce an inapparent, immunizing infection. This was found to be impractical, for dosage-range between a fatal and an inapparent infection was entirely too narrow. Passive-active immunization was next tried and the unexpected results of both passive and passive-active immunization experience are reported here, together with the more expected type of results from use of a formalinized vaccine preparation. Also included in this paper are studies on effect of cortisone injections subsequent to immunization, on immunity previously acquired.

Materials and methods. A strain of WN virus obtained a number of years ago through the courtesy of Dr. Smithburn was used in its 30th mouse passage, prepared as a pool of frozen brain suspension. This pool had a titer of $10^{-8.6}$ when tested intracerebrally in mice and $10^{-8.5}$ subcutaneously in hamsters. Syrian hamsters from Lakeview Hamster Colony, N. J., were used when 8 to 10 weeks of age for the beginning of immunization procedures. Antisera were prepared in rabbits and in hamsters and their neutralization indices determined by the intracerebral mouse neutralization test(4). All sera were injected intraperitoneally, suitably diluted in a 0.5 cc volume in the hamsters to be protected. The selected virus dilution was given subcutaneously in a volume of 0.1 cc. Vaccine was prepared by mixing a freshly prepared 20% infected mouse brain suspension with an equal volume of 0.4% formalin. The mixture was kept at room temperature for a week with frequent agitation, then kept at +4°C in the refrigerator. A test to demonstrate the absence of detectable live virus was then made by intracerebral inoculation of mice, undiluted and at 10^{-1} after neutralization of the formalin.

Exp. 1. Passive-active immunization using one dose of WN immune rabbit serum. Immune rabbit serum with a neutralization index of 15,000 was employed in 5-fold serial dilutions ranging from 1:5 through 1:625. Each dilution was inoculated into each of a group of 6 hamsters. Twenty-four hours after serum inoculation each ham-

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TABLE I. Response to Subcutaneous Injection with 100 LD_{50} of WN Virus after Passive Immunization with WN Hyperimmune Rabbit Serum.

Serum dilution	Days of death	Mortality ratio
1: 5	27, 29	2/6
1: 25	14, 14, 16, 16	4/6
1: 50	15, 15, 15, 17, 17, 18	6/6
1: 75	9, 11, 12, 14, 14, 15	6/6
1:100	9, 10, 10, 10, 11, 12	6/6
1:125	9, 10, 10, 11, 11, 12	6/6
1:625	6, 8, 9, 9, 9, 10	6/6
Control	6, 7, 8, 8, 9, 10	6/6

ster was inoculated subcutaneously with 100 hamster LD_{50} of WN virus. An additional group of 6 hamsters was inoculated with the virus only. Five weeks after the virus inoculation the surviving hamsters were challenged subcutaneously with 100 LD_{50} of WN virus.

There was some degree of protection against the 1st injection of live virus in the group of hamsters which received the 1:5 dilution of serum and a small degree of protection in the group which received the 1:25 dilution, while there was no reduction of fatality in the other groups. (Table I) However, the immune serum prolonged the interval before death, and the greater the concentration of serum the more prolonged the period. This prolongation of life is observed even with the 1:125 serum dilution. Live virus challenge after 5 weeks, of the hamsters surviving from the 1:5 and 1:25 serum dilutions, gave the following results: 2 out of 4 hamsters died from the 1:5 serum and 0 of 2 died from the 1:25 group. This method did not appear to offer encouragement for producing passive-active immunization but it was felt that if a 2nd dose of serum was given at a suitable interval, many hamsters might survive among those whose lives were only prolonged in this experiment.

Exp. 2. Passive-active immunization using 2 doses of WN immune rabbit serum. Three groups of 5 hamsters each were treated in the following manner: In Group I each hamster received on the 1st day a 1:25 dilution of the same immune serum used in Exp. 1. On the 2nd day each was inoculated with 100 LD_{50} of WN virus as in the previous experiment. In Group II each hamster received serum and virus in a quantity and manner identical with Group I, but in addition was given another dose of serum in a 1:25 dilution 1 week after the virus inoculation. In Group III each hamster received virus only.

TABLE II. Response to Subcutaneous Injection with 100 LD_{50} of WN Virus after Passive Immunization with One and 2 Doses of WN Hyperimmune Rabbit Serum Diluted 1:25.

Serum doses	Days of death	Mortality ratio
1	15, 18, 18, 19	4/5
2	12, 12, 13, 20	4/5
0	8, 9, 9, 10, 10	5/5

This method of 2 doses of WN hyperimmune serum also failed to provide suitable passive-active immunization (Table II). The 2 surviving animals were not challenged. It was considered possible that the cause of failure of these methods was due to the rapid destruction and excretion of heterologous serum. WN hyperimmune hamster serum was then prepared in the hope of getting better results because of its lower rate of excretion.

Exp. 3. Passive-active immunization using WN immune hamster serum and a 10^{-1} dilution of virus. This experiment was done in a manner similar to that of Exp. 1, with an exception which was an oversight. They were inoculated with 0.1 cc of a 10^{-1} dilution of WN virus instead of a 100 LD_{50} dilution. Observation was continued in spite of this fact. WN hamster immune serum with a neutralization index of 870 was used. The serum dilutions employed are shown in Table III. Each group was composed of 10 animals. Three hamsters from each group were bled out just before virus inoculation of the remainder and these sera were titrated for WN neutralizing antibodies using the intracerebral mouse test. From the table it can be seen that the results in respect to survival were not

TABLE III. Response to Subcutaneous Challenge with 0.1 ml of 10^{-1} WN Virus after Passive Immunization with WN Immune Hamster Serum.

Serum dilution	Days of death	Mortality ratio
1: 5	9, 12, 15, 16	4/7
1:25	7, 9, 10, 10, 16	5/7
1:50	9, 10, 11, 11, 14	5/7
Control	8, 8, 9, 9, 9, 10, 10	7/7

TABLE IV. Log Neutralization Index* to WN Virus in Sera of Hamsters Sacrificed 24 Hr after Intraperitoneal Inoculation of Hamster Immune Serum.

Serum dilution	Log neutralization index: Hamster 1	Hamster 2	Hamster 3	Mean
1: 5	.9	1.0	2.0	1.3
1:25	.8	.8	1.1	.9
1:50	.3	.9	.7	.6

* Intracer. mouse test.

more encouraging than those of the earlier experiments using rabbit serum. It was thought that this result was quite possibly due to the use of a high concentration of virus. Results on serum neutralization tests (Table IV) suggest that antibody was present in the blood at a detectable level 24 hours after immune serum inoculation of the 1:5 and the 1:25 dilutions. The very few survivors (Table III), when subsequently challenged with 1000 hamster LD_{50} of WN virus 4 weeks after the 1st virus inoculation, were found to be resistant to disease.

Exp. 4. Passive-active immunization using one dose of WN immune hamster serum and 100 LD_{50} of virus. This experiment was done in a manner similar to the previous experiment, with the exception that 100 hamster LD_{50} of WN virus were used for inoculation. The serum dilutions employed are shown in Table V. Each group was composed of 10 animals.

The results of this experiment were no more encouraging than those of the earlier experiments. There were only 8 survivors, simply a slight prolongation of life in most instances. These remaining 8 hamsters survived when challenged with 1000 hamster LD_{50} WN virus 4 weeks after the 1st virus inoculation. From the results of the different experiments on passive-active immunization it was found that this method of immunization was impractical as a method of obtaining large numbers of surviving immune animals and active immunization of hamsters was tried.

Exp. 5. Active immunization of hamsters to WN virus. Two groups of hamsters were employed. The 1st group received 1 injection of 0.5 cc of WN formalin inactivated vaccine intramuscularly, the 2nd group received 2 injections each of 0.5 cc of vaccine intramuscularly 2 weeks apart. Three weeks after the last vaccination or after the 1st in those receiving only 1 dose, the hamsters were inoculated subcutaneously with 100 subcutaneous hamster LD_{50} of WN virus to provide immunization with active virus in a manner which, it was hoped, would lead to survival of most animals.

From Table VI it can be seen that 2 doses of vaccine gave complete protection to 100 LD_{50} hamster doses of WN virus. Hamsters immunized with 1 dose of vaccine had a lower survival rate.

In a later experiment (5) in which 500 hamsters were basically immunized to WN virus, to be challenged later with JBE virus, the hamsters were inoculated each with only 1 dose of 0.5 cc of another lot of similarly prepared WN vaccine. One week later each hamster was given 100 hamster LD_{50} of WN virus subcutaneously. Only 5 out of the 500 hamsters died, *i.e.*, about 1% deaths. Serological tests done on a pool of sera of 5 of the surviving hamsters showed a complement fixing titer of 1:16 and a neutralization index of 1.9.

Exp. 6. Effect of cortisone on pre-existing active immunity. Fourteen hamsters were actively immunized to WN virus by 2 injections of WN vaccine given 1 week apart. Two weeks after the last injection each hamster was inoculated subcutaneously with 1000

TABLE V. Response to Subcutaneous Inoculation with 100 LD_{50} WN Virus after Passive Immunization with WN Immune Hamster Serum.

Serum dilution	Days of death	Mortality ratio
1:10	10, 11, 12, 13, 13, 13, 14	7/10
1:20	9, 9, 10, 10, 10, 11, 13, 13	8/10
1:30	9, 10, 10, 10, 10, 11, 11, 13, 13	9/10
1:40	7, 7, 7, 8, 9, 9, 13, 13	8/10
Control	7, 7, 7, 8, 8, 8, 9, 9, 10, 10	10/10

TABLE VI. Response of Hamsters Immunized with One and 2 Injections of WN Vaccine and Inoculated Subcutaneously with 100 LD_{50} Hamster Doses of WN Virus.

Vaccine doses	Mortality ratio
1	3/10
2	0/10
0	5/ 5

TABLE VII. Effect of Cortisone on Previously Acquired Active Immunity of Hamsters.

Immunization	Cortisone treatment	WN virus inoc.	Mortality ratio
WN	Cortisone	Virus	1/4
WN	None	"	0/4
None	Cortisone	None	1/4
"	None	Virus	4/4

hamster LD_{50} of WN virus, and 2 weeks later each was inoculated with 10,000 hamster LD_{50} of the virus. These hamsters were divided into 2 groups of 7 each. Three hamsters were bled out from each group and their sera pooled and titrated to determine the neutralization index to WN virus. The 1st group of 4 remaining hamsters was treated with cortisone in a dose of 0.25 mg/g body weight (intentionally larger than the standard dose) given over 4 days and challenged subcutaneously with 300,000,000 hamster LD_{50} (0.1 cc of 10^{-1} dilution) of WN virus on the 2nd day of the experiment in a manner previously described(*2*). The 2nd group of 4 remaining animals was challenged with virus only. A control group of 4 non-immune hamsters was treated with the same dose of cortisone only, and a 2nd control group of 4 normal hamsters was inoculated with the same dose of the virus only. The hamsters were watched for a period of 3 weeks after challenge and then the surviving ones were bled and their sera were titrated separately for neutralizing antibodies.

Only 1 hamster from the immunized group which received cortisone died on the 6th day after virus inoculation. (Table VII). The autopsy done on that animal showed that the liver was mottled and friable and the spleen had many septic nodules. Culture of liver, spleen and blood on blood agar plates revealed hemolytic streptococci. The brain suspension of that hamster, after penicillin and streptomycin were added, was negative for bacterial growth and negative for virus when inoculated intracerebrally in mice. It was thus clear that this hamster did not die from the WN virus, but from bacterial infection, probably due to the high dose of cortisone given. Virus control animals all died as expected between the 7th and the 9th days. One cortisone control animal died of sepsis as in the immunized group. Thus there was no evidence of cortisone having interfered with the pre-existent immunity of the immunized group. Results of neutralization tests (Table VIII) reveal that there was no significant difference in the level of neutralizing antibodies between the cortisone treated and the non-cortisone treated immune groups post challenge. In fact, since there was no significant antibody rise in either group 3 weeks after challenge, it appears that all the hamsters were probably immune to the extent of resisting infection as well as disease.

Discussion. In an attempt to immunize hamsters to WN virus, both passive-active and active methods of immunization were tried. In passive-active immunization WN rabbit immune serum was first tried in 1 dose. Results were unsatisfactory in that there were only a few survivors even with the least diluted serum. In the higher dilutions only the incubation period was prolonged. Some hamsters surviving this method of immunization were found not to be immune when challenged later with live WN virus. In the case of the lowest serum dilution the serum apparently neutralized the virus and no infection took place; thus, at the end of the immunization period the hamsters were not immune. In the next higher serum dilution the small percentage of surviving hamsters was found later to be immune to live WN virus. The low survival rate during attempted active-passive immunization was first attributed to the fact that heterologous serum is rapidly destroyed and excreted. Two doses of serum were then employed, but this also was found to be unsatisfactory. Homologous serum was used

TABLE VIII. Effect of Cortisone on the Titer of Neutralizing Antibodies in Sera of Actively Immunized Hamsters after Virus Challenge.

	Log neutralization index*					
Cortisone treatment	Pre-challenge pool	Hamster No. 1	2	3	4	Mean†
Yes	3.1	2.8	3.6	3.0	—‡	3.1
No	3.1	3.3	3.2	3.2	3.7	3.3

* Intracer. mouse test.
† Geometric mean.
‡ This animal died from sepsis.

with the same poor results in so far as obtaining large numbers of survivors. However, in the dosages of virus and serum employed the few survivors did resist subsequent virus challenge.

In active immunization better results were obtained especially when 2 doses of formalin inactivated virus vaccine were used, followed 3 weeks after the last dose by 100 hamster LD_{50} of WN virus. Later(5), using 500 hamsters, it was found that even 1 dose of another lot of WN vaccine followed 1 week later by 100 hamster LD_{50} of WN virus provided a rapid and satisfactory method of immunization of hamsters to WN virus, with only 1% mortality during the procedure.

Since the principal purpose of this work was to prepare animals for subsequent subcutaneous challenge with certain other group B encephalitis viruses such as JBE and SLE which required cortisone treatment to render them susceptible, it appeared necessary to investigate the effect of cortisone on any preexisting actively acquired immunity. Kilbourne(6), working with influenza virus, demonstrated that mice with previously existing actively acquired immunity, when challenged with active virus and given large amounts of cortisone were rendered somewhat less immune. Even with smaller doses of cortisone, although most of the challenged mice survived, virus was found to persist in the lungs for 7 days while none was found in the lungs of those not receiving cortisone. Our tests with a small group of actively immunized hamsters treated over 4 days with such large doses of cortisone that 1 out of 4 from each of 2 groups died of bacterial sepsis, and challenged with very large doses of virus ($10^{8.5}$ LD_{50} when administered by the same route in normal hamsters) gave evidence of complete protection against the virus. Possibly, if less completely immunized animals had been used some quantitative differences might have become apparent. Further evidence indicating that the animals were even protected against inapparent infection from the large virus challenge together with cortisone is indicated by finding that there was no significant rise of neutralizing antibodies 3 weeks after virus was given in either the normal or cortisone treated animals. We did not test for persistence of virus as did Kilbourne. It is possible that our test demonstrating a lack of antibody rise should not be interpreted as lack of infection for cortisone would suppress this temporarily. However, we found in other work(2) that suppression of antibody rise by cortisone was not complete and did not appear to persist for long. Thus, since no rise was observed after 3 weeks, we believe it probable that infection was actually prevented.

Summary. Both passive-active and active methods of immunization of hamsters to WN virus were tried. The passive-active method gave very poor results; the active method gave satisfactory results. A procedure in which the hamsters are given 1 or 2 doses of 0.5 cc of freshly prepared WN vaccine, followed 1 week later by 100 hamster LD_{50} of WN virus, produced a satisfactory and rapid method of immunization. Hamsters immunized by vaccine plus active virus, when given large doses of cortisone and challenged with $10^{8.5}$ LD_{50} of virus survived. These hamsters and similar immunized control animals not given cortisone had all failed to develop increased neutralizing antibody titers 3 weeks following the virus challenge. These results are interpreted to indicate that cortisone probably did not substantially reduce previously acquired immunity to disease or to infection.

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Myotrophic (Anabolic) Activity of 4-Substituted Testosterone Analogs. (23105)

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The screening in castrated rats of a group of new steroids recently synthesized in this laboratory(1) showed that some of the substituted testosterone analogs increased the weights of levator ani muscle without modifying to any great extent that of the ventral prostate. This suggested that an anabolic effect was occurring and induced us to investigate the biologic activity of these steroids. Thus, in the absence of potent androgenic activity they may have therapeutic value as anabolic agents.

Method. Myotrophic and androgenic activity were determined by the method of Herschberger, Shipley and Meyer(2) using groups of 7 or more previously castrated albino rats weighing 30-40 g. The steroids were either prepared in oil or as aqueous suspensions with 0.5% carboxymethylcellulose, 0.4% Tween 80, 0.9% benzyl alcohol and physiological salt solution and injected subcutaneously daily for 7 days. Twenty-four hours after the last injection, the animals were autopsied, the levator ani muscle, ventral prostate and seminal vesicles were removed and their wet weights recorded. In preliminary studies, the dry weights were also recorded and since there was no evidence of hydration, the wet weights were used throughout the experiment. For comparing the anabolic and androgenic activity, the following ratio was established:

$$\frac{\text{Exp. levator ani wt} - \text{Control levator ani wt}}{\text{Exp. prostate wt} - \text{Control prostate wt}}.$$

Other hormonal activities were evaluated as follows: life maintaining(3), glycogen deposition and Na retention(4), anti-inflammatory and water diuresis(5), proinfectivity(6), progestational(7) and estrogenic(8).

Results. I. *Myotrophic and androgenic activity of the new steroids.* The effects produced by various doses of the testosterone analogs are shown in Table I and are compared with those due to testosterone propionate, methylandrostenediol and 19-nor-17-α-ethyltestosterone. All weights differ significantly from those of the controls ($P<.01$). The data show that 4-chloro analogs (4-chlorotestosterone acetate, 4-chloro-19-nortestosterone acetate or cyclopentyl propionate, and 4-chloro-17-α-methyl-19-nortestosterone) and 4-hydroxy-19-nortestosterone acetate produce myotrophic effects similar to that of testosterone propionate. The average weights of the levator ani muscle following these agents are comparable. Investigation of the androgenic activity shows that the 4-chloro analogs have weak stimulating action on prostate and seminal vesicles whereas testosterone propionate and methylandrostenediol increase weights of the accessory sex organs of male rats. The weight of the prostate following 4-chlorotestosterone acetate or 4-chloro-19-nortestosterone acetate is significantly less ($P<.01$) than that due to testosterone or methylandrostenediol. These analogs, therefore, have therapeutic indexes (levator ani wt: ventral prostate wt) which exceed those of testosterone or methylandrostenediol. Since similar results were obtained with 19-nortestosterone cyclopentyl propionate, it appears that the presence of the chlorine atom at C-4 or the absence of the 19-methyl group decreases androgenic potential of testosterone without reducing the myotrophic action. Thus, the 4-chloro-19-nortestosterone acetate or the cyclopentyl propionate which show both changes in the same molecule have the highest indexes.

As to the mechanism of 4-substitution, it is noteworthy that 4-hydroxytestosterone is a myotrophic agent but has less androgenic potency than testosterone. This results in an elevated index. On the other hand, 4-substitution with Br or F results in less active analogs.

II. *Other hormonal effects of 4-chloro analogs.* Neither 4-chlorotestosterone nor 4-

TABLE I. Myotrophic (Anabolic) and Androgenic Activity of New Testosterone Analogs.

Treatment	No. rats	Dosage (μg/day)	Mean wt in mg ± S.E.* Levator ani	Ventral prostate	Seminal vesicles	Therapeutic index (lev. ani/ prostate)
Castrated controls	174		8.6 ± .33	9.3 ± .41	5.4 ± .48	—
Testosterone propionate	52	100	22.2 ± 1.05	62.5 ± .81	50.6 ± 1.78	.26
	19	250	27.9 ± 2.22	87.1 ± 6.72	73.4 ± 3.27	.25
	25	500	31.3 ± 1.73	91.9 ± 4.17	87.9 ± 4.20	.27
Methylandrostenediol	7	"	23.5 ± 1.51	63.7 ± 3.70	53.1 ± 4.66	.28
19-nortestosterone cyclopentyl propionate	19	"	40.8 ± .57	59.1 ± 2.71	59.3 ± 2.59	.65
19-nor-17α-ethyltestosterone	7	"	26.2 ± 3.39	41.0 ± 5.66	37.1 ± 3.99	.55
4-fluorotestosterone acetate	7	"	14.0 ± 1.50	20.2 ± 1.46	8.3 ± .81	.50
4-chlorotestosterone acetate†	37	100	11.5 ± .84	20.6 ± 1.47	9.4 ± .53	.26
	27	250	26.7 ± 1.69	35.7 ± 1.50	20.4 ± 1.01	.68
	56	500	37.2 ± 1.31	55.7 ± 1.43	44.3 ± 1.61	.62
4-chlorotestosterone propionate	7	"	27.4 ± 2.52	43.1 ± 3.19	40.1 ± 7.30	.56
4-bromotestosterone	12	"	14.4 ± 1.20	25.9 ± 1.45	16.7 ± 1.40	.34
4-chloro-11β-hydroxytestosterone acetate	7	"	21.1 ± .64	41.4 ± 1.79	31.7 ± 1.30	.39
4-hydroxytestosterone acetate	7	"	20.5 ± 4.98	32.0 ± 4.68	25.2 ± 3.12	.52
4-chloro-19-nortestosterone acetate	23	100	21.2 ± 1.25	20.4 ± 1.20	12.8 ± 1.26	1.13
	10	300	27.1 ± 2.25	26.7 ± 1.26	30.4 ± .97	1.06
	8	500	34.1 ± .96	31.1 ± 1.96	21.9 ± 1.64	1.16
4-chloro-19-nortestosterone cyclopentyl propionate	15	100	11.2 ± 1.35	12.5 ± 1.34	7.9 ± .60	.81
	18	250	27.1 ± 2.12	18.4 ± 1.67	14.8 ± 1.69	2.03
	8	500	27.3 ± 3.31	18.2 ± 1.53	19.6 ± 1.28	2.10
4-hydroxy-19-nortestosterone acetate	7	"	29.6 ± 1.13	47.7 ± 3.80	54.0 ± 4.65	.55
4-chloro-17α-methyl-19-nortestosterone	7	"	24.7 ± 2.03	31.2 ± 1.15	34.6 ± 3.12	.73

* Stand. error $= \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$. † Aqueous suspension.

chloro-19-nortestosterone has estrogenic or corticoid activity. Neither increases glycogen content of the liver nor affects the survival rate of adrenalectomized rats. Moreover, they do not cause Na retention, K loss, increase output of urine or aggravate experimental infection. Although the former has no progestational action, the latter is about ½ to ¼ as active as progesterone.

Summary. 1) C-4 substituted testosterone analogs possess potent myotrophic and weak androgenic activity as measured by increase in weight of levator ani muscle and ventral prostate gland, respectively. 2) Daily subcutaneous injections of 0.5 mg 4-chlorotestosterone acetate induce greater anabolic and lesser androgenic effects than similarly administered testosterone propionate; the anabolic/androgenic ratios are 0.62 for the former and 0.27 for the latter. 3) The anabolic activity of the 4-chloro-19-nortestosterone acetate and 4-chloro-19-nortestosterone cyclopentyl propionate is equivalent to that of testosterone propionate, but their effect on prostate growth is less. The anabolic/androgenic ratios are 1.16 and 2.10 respectively. 4) Neither 4-chlorotestosterone actetate nor 4-chloro-19-nortestosterone acetate has estrogenic or corticoid activity. Only the latter, however, has progestational activity.

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A Modified Seroflocculation Reaction in Relationship to Invasive Neoplasms.* (23106)

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In a previous communication(1) a seroflocculation reaction was described as a laboratory aid in diagnosis of invasive cancer in humans. This reaction consisted of mixing serum with ethyl choladienate under controlled conditions and analyzing the resulting flocculation reaction. While a preponderance of positive reactions was obtained with sera from histologically proven malignancies, a considerable number of positive reactions was given by sera from normal individuals and from some patients with non-malignant pathological conditions. This is to report on a modification in the procedure whereby the incidence of "non-specific" positives can be significantly reduced. As a result of an investigation by Kidd(2), who noted that non-specific reactions to the Brown-Pearce tumor antigen by normal rabbit sera could be eliminated through heating the sera at 65°C for 30 minutes, the following study was made to determine whether a similar treatment of human sera would reduce the incidence of non-specific positives in the flocculation test for malignancies. Preliminary studies, in which sera were heated at various temperatures ranging from 56°C to 65°C, showed that 64°C was optimum. Higher temperatures tended to coagulate certain sera. Sera from 2,747 individuals were tested (Table I); 220 of these individuals had biopsy proven cancer (Table II), 1,822 were normal blood donors, and 705 had non-malignant diseases (Table III). The results clearly indicate that this modified procedure reduces the number of false positives.

Materials and methods. Blood was obtained by venipuncture under aseptic precautions and stored overnight at temperatures between 4°-6°C. Serum was separated from the clot by decantation and centrifugation. Each serum was then divided into approximately 2 equal volumes. One was heated at 56°C and the other at 64°C for 30 minutes simultaneously. *For testing, all sera and solutions were maintained at 15°C (iced water bath).* The following solutions were required: (A) Buffered saline solution(3) consisting of 6.5 ml of 0.1M citric acid, 43.5 ml of 0.2M anhydrous disodium phosphate, 42.5 ml of 5% sodium chloride and then diluted to 250 ml with water; (B) 0.1 mg percent of bovine albumin solution (Fraction V Armour Laboratories) in buffered solutions; (C) 1% cholesterol in absolute ethanol; (D) Ethyl choladienate 15 mg per ml in 95% ethanol. The *antigen suspension* was prepared by depositing at the bottom of a tube 0.013 ml of the cholesterol solution (C), plus 0.1 ml of the antigen solution (ethyl choladienate solution D) and mixed by rapid rotation of the tube. To the alcoholic solutions C and D was added 0.4 ml of the citrate phosphate buffered nor-

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TABLE I. Analysis of Cases Studied.

Category	Total No. of cases	Positive reactions with heated serums 56°C	64°C
	2747		
		(%)	(%)
Carcinoma	220	204 (93)	202 (92)
"Normal" individuals and blood bank sera	1822	238 (13)	11 (0.6)
Non-malignant hospitalized cases	705	275 (38)	71 (10)

mal saline containing bovine albumin (B). These were thoroughly mixed by gently inverting the tube several times. (Too rigorous agitation may precipitate suspension.) The suspension was used within 15 minutes after preparation. The test was carried out as follows: All glassware was thoroughly cleansed, rinsed in distilled water and oven-dried. Two tubes (13 x 100 mm) were used. To one was added 0.1 ml of serum and 0.15 ml of antigen suspension; to the other 0.07 ml of serum and 0.15 ml of antigen suspension. Rack was shaken in Kahn shaker for 3 minutes. To each tube was then added 0.8 ml of buffered normal saline (A), and centrifuged at 3,000 rpm for 10 minutes. Readings were made in diffuse standardized light after shaking tubes to disperse precipitate at the bottom. Positive reactions gave particles in a crystal clear medium in one or both tubes. Negative reactions gave various degrees of turbidity with or without particles. Occasionally there occurred a very slight turbidity in the test serum as compared with the sparkling clarity of the positive control. These "doubtfuls" were considered negatives. Sera were tested in units of thirty. In each unit was included a known positive and a known negative control.

Results. Comparison of the earlier and the present modified methods (Table I) shows a striking reduction in the incidence of "false positives." Tests on the normal group ranging from 20 to 70 years of age, clearly demonstrated the reduction of "false positives" (13% to 0.6%) by heating the serum at 64°C. The results also show that heating at 64°C did not diminish sensitivity of the reaction with respect to detection of malignancies. It should be noted that this group of malignant tumors does not contain carcinomas *in situ*. The seroflocculation reaction has been concerned primarily with invasive malignancies. The limited number of neoplasms of female sex organs and none in children is due to the fact that the sera were obtained almost exclusively from the male hospital wards.

Occurrence of positive reactions in a wide

TABLE II. Cancer Sera.*

Site of malignancy	Total cases	Reactions with heated serums 56°C No. positive	56°C No. negative	64°C No. positive	64°C No. negative
	220	204	16	202	18
Head and neck	41	40	1	40	1
Chest	28	28	0	28	0
Gastro-intestinal	34	33	1	33	1
Genito-urinary	45	43	2	42	3
Bone	10	9	1	9	1
Female breast	7	5	2	4	3
" pelvic	3	2	1	2	1
Metastasis, primary unknown	3	3	0	3	0
Lymphoma	9	9	0	9	0
Squamous-cell epithelioma	21	17	4	18	3
Basal-cell epithelioma	14	11	3	10	4
Melanoma	5	4	1	4	1
% positive		93%		92%	

* All diagnoses based on histopathological findings exclusively.

TABLE III. Non-Cancer Serum.

Diagnosis	Total cases	Positive reactions with heated serums 56°C	64°C
	705	275	71
Rheumatoid arthritis, active	96	38	6
Endocrine disturbances + medication	143	59	16
Allergy disease + medication	113	32	12
Cirrhosis	14	10	4
Gastric ulcer	30	11	3
Duodenal "	13	3	0
Active tuberculosis	25	16	4
Bronchiectasis	7	4	0
Diabetes *on* insulin	66	33	7
" *no* "	46	10	2
Pregnancy	14	6	1
Chronic cystic mastitis	7	2	1
Menorrhagia	31	19	2
Infection with temperature	20	12	10
Keratosis	26	4	0
Bone cyst	4	0	0
Lipogranuloma	2	1	0
Benign prostatic hypertrophy	48	15	3
% positive		39%	10%

variety of other pathological conditions (non-malignant group) was also considerably diminished (38% to 10%). The persistence of positive reactions in this group might suggest the presence of some change in plasma which is similar to that occurring in malignancies.

Summary. A method is described for an improved laboratory aid in mass screening for invasive neoplasms. The modification of the described seroflocculation test consisted essentially in heating sera at 64°C for 30 minutes.

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Transfer of Lymph Node Cells to Recipient Rabbits Pre-Injected with Blood Leucocytes of Donors.*† (23107)

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Studies of antibody formation in this laboratory have recently involved the technic of transfer of lymph node cells from donor to recipient animals, as described by Chase (1,2). In earlier studies, cells to be transferred were obtained from lymph nodes draining sites of injection of antigen into donor rabbits(3,4); more recently lymph node cells obtained from uninjected donors (or from donors injected with heterologous antigens) were incubated *in vitro* with the antigenic substance and then transferred to X-irradiated recipient rabbits(5,6). In each case antibody to the antigen referred to appeared subsequently in sera of recipients. In both systems it was found that living cells were necessary, and that antibody did not appear in recipients of injured cells. In studies with other systems involving humoral antibody, or resistance to tumor homografts, various workers also found that injury to lymph node cells prior to transfer results in failure of antibody to appear in the recipient(1,2,7-9). However, the role of transferred cells in the formation of the antibody subsequently found in the recipient has not been clarified. In the present study the relation of transferred cells to such antibody was approached by attempts to alter the host-tissue environment of the transferred cells, rather than by manipulation of the cells themselves. This approach was suggested by

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the work of Medawar(10,11) who showed that after rejection of a skin homograft from a given donor by a given recipient, a second skin graft from the same donor to the same recipient was rejected in a substantially shorter period of time (the "second-set phenomenon").

In the present study experiments were carried out in the lymph node cell transfer system, in which some of the recipient rabbits were injected with blood leucocytes obtained from donor rabbits, prior to lymph node cell transfer, and the curves of antibody concentrations with time after transfer were compared in the two groups of rabbits.

Materials and methods. Pre-injection of donor blood leucocytes. Adult rabbits scheduled to become donors of lymph node cells were bled by cardiac puncture. The heparinized blood was mixed with one volume of 3% gelatin solution in tall narrow tubes, and the mixture allowed to stand one hour at room temperature. The layer above the sedimented erythrocytes was removed by suction, and the leucocytes contained therein were collected by centrifugation. The cell sediment was suspended in a convenient volume of Tyrode's solution containing 7½% normal rabbit serum and heparin. Recipient rabbits were injected intradermally in the back in 6-8 sites. *Cell transfer.* The popliteal and axillary lymph nodes of donor rabbits were excised and cells obtained from them by technics described elsewhere(6). The lymph node cells were incubated *in vitro* with filtrates of trypsin-treated suspensions of dysentery bacilli as described earlier(12). After ½ hour incubation at 37°C, the lymph node cells were washed and transferred to X-irradiated recipients by intravenous route. Thereafter recipients were bled at regular intervals and their sera tested for agglutinins to dysentery bacilli. No recipients were irradiated at time of pre-injection of donor leucocytes, but all were given 425r of deep X-ray treatment 24 hours before transfer of incubated lymph node cells.

Results. Effect of injection of donor leucocytes; variations in time interval and dosage. In some experiments recipients were injected intradermally with blood leucocytes from

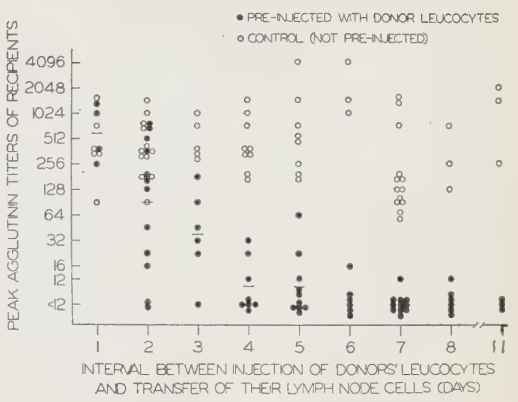

FIG. 1. Peak agglutinin titers of individual recipients of lymph node cells incubated *in vitro* with filtrates of trypsin-treated suspensions of dysentery bacilli. Recipients in the experimental group had been inj. intradermally with donor leucocytes at intervals, as indicated, prior to transfer of the incubated lymph node cells. The bar indicates geometric mean titer of group of pre-inj. recipients.

prospective donors several days before cell transfer. On day of transfer, lymph node cells of these donors were incubated *in vitro* with filtrates of trypsin-treated suspensions of dysentery bacilli. These cells were washed and transferred to irradiated recipients which had been pre-injected at various times (1-11 days earlier), and also to control recipients not pre-injected. The results of such experiments, summarized in Fig. 1, show that whereas control recipients developed agglutinin titers in the usual range, recipients pre-injected with leucocytes on fourth to eleventh day before transfer showed only very low titers of agglutinin. Recipients which had been pre-injected 3 or 2 days before transfer showed some reduction in agglutinin titer, and serum titers of those which had been pre-injected one day before transfer were indistinguishable from controls which had not been pre-injected. In most of these experiments the number of leucocytes pre-injected was 10 million. In the first experiments the number of donor blood leucocytes injected into recipients ranged between 30 and 100 x 10^6. In experiments each involving the range of 100 x 10^6 to 0.1 x 10^6 an injection of approximately 10^6 leucocytes was adequate to produce the effect described when injected 6 days prior to cell transfer.

TABLE I. Peak Agglutinin Titers of Recipient Rabbits Pre-Injected with Various Blood Cells.

	Control rabbits, not pre-inj.	Recipient rabbits pre-inj. with: Blood leucocytes of— Rabbit	Man	Horse	Cow	Chicken	Rabbit erythrocytes
	256	64	64	256			384
		<12	16	64			384
		<12					16
	96	12					48
	96	<12					48
		<12					
	128	<12	384				512
		<12	32				192
		16	24				32
			24				
	192	12			384	384	768
	192	<12			192	384	384
	768				128	192	128
	192				128	128	96
					96	24	
	768	<12	256	384			1024
	384	<12	64	384			512
	128	<12	16	256			384
	96	12	<12	128			96
	64			96			
Geometric mean titer of group ($\log_2$)	7.6	3.0	5.3	7.5	7.3	7.2	7.4

Peritoneal exudates were obtained from donor rabbits 2 days after the intraperitoneal injection of either of 2 irritants: heavy mineral oil, which yielded an exudate predominantly of monocytes (75-81%), or an emulsion of lanolin and light mineral-oil in saline, which yielded a cell population largely of polymorphonuclear leucocytes (60-78%). The cells thus obtained, as well as leucocytes obtained from blood, were injected intradermally into recipient rabbits (approximately 10^7 cells/animal) 7 days before cell transfer. Recipients pre-injected with cells of either type of peritoneal exudate failed to develop agglutinins or did so in low titer, the distribution of titers being similar to that in the group of recipients pre-injected with leucocytes obtained from blood. The group of recipients not pre-injected developed a mean peak titer of 768.

Pre-injection of rabbit erythrocytes or of blood leucocytes of other animal species. Leucocytes prepared from chicken, cow, horse, and human blood, as well as erythrocytes and leucocytes from rabbits, were injected intradermally into prospective recipients 6 days before cell transfer. As can be seen in Table I, following transfer of incubated lymph node cells, the recipients which had been pre-injected with leucocytes obtained from rabbit blood developed no measurable amounts of agglutinin, or very low titers. On the other hand, of 16 recipients pre-injected with rabbit erythrocytes, 9 developed agglutinin titers as high or higher than control recipients, 6 developed somewhat lower titers and one developed a barely detectable level. The geometric mean of the maximum titers of the whole group of recipients was similar to the controls. Recipients pre-injected with leucocytes obtained from blood of chickens, cows and horses also developed agglutinin titers similar to those of the control (non-pre-injected) recipients. Of 10 recipients pre-injected with leucocytes obtained from human blood, the sera of 2 showed agglutinin titers in the range of control recipients, 6 were lower and 2 were barely detectable. The geometric mean titer of this group was distinctly lower than that of the control group.

Discussion. In sera of control rabbits of above experiments, as in recipient rabbits of earlier studies, agglutinins to dysentery bacilli were found after transfer of lymph node cells

incubated *in vitro* with antigenic material derived from those organisms. In the present study recipients which had been pre-injected intradermally with leucocytes from donor animals did not develop agglutinins after transfer of incubated lymph node cells. This failure of appearance of antibody is presumably due to an alteration, brought about by pre-injection of donor leucocytes, in the environment of the transferred cells within the recipient. The following data are consistent with an hypothesis that this alteration is due to an immunologic effect:

1. *Rabbit erythrocytes.* The geometric mean antibody titer of recipients pre-injected with donors' erythrocytes was similar to that of the control group. (It is felt that the occurrence of lower peak titers in some animals of this group may have been due to leucocytic contamination of the erythrocyte suspension, which was generally more than one/thousand rbc.)

2. *Heterologous leucocytes.* The appearance of antibody in recipient rabbits was not affected by pre-injection of leucocytes obtained from cow, chicken or horse. In the case of human leucocytes there was evidence of reduction in antibody concentration in sera of more than half of the recipients. This suggests the possibility of a similarity in structure between some antigen(s) in human and rabbit leucocytes.

3. *Time relations of pre-injection to cell transfer.* An interval of 2-3 days between pre-injection and cell transfer was required for substantial suppression of antibody formation. This period, added to the 3-day interval after the transfer of *in-vitro*-incubated lymph node cells before antibody would normally appear, yields a total of 5-6 days, an interval which would be consistent with that required for an appreciable immunologic reaction of the recipients' tissues to antigens in leucocytes of donors. While the above data suggest an immunologic mechanism for this suppression of cell-transfer effect, the data available thus far have not proven that an immunologic mechanism is involved, or indicated its nature.

The observations reported here are parallel to those of Medawar(10) and of others in tissue homografting, in that the response of the recipient is different according to whether it is involved in the first or second contact with the tissue (or cells) of the donor, and that the effect of first contact could be brought about by pre-injection of leucocytes from the donor and not by erythrocytes(11).

There is a difference, however, between the phenomena observed in the 2 kinds of experiments. In one case (skin homograft) the second graft is rejected earlier; in the other case (cell transfer) antibody fails to appear. This probably reflects the difference in the 2 experimental situations. In the case of a second skin graft the interaction between the grafted cells and the immunologic agent in the host tissue must await vascularization of the graft; death of the grafted tissue follows within a few days. In the lymph node cell transfer cells are injected directly into the blood stream, so that injury to these cells could occur within a short time. Such injury might well result in complete failure of antibody to appear, since antibody does not normally appear until the third day after cell transfer (in the present system).

The experiments described above give further evidence for some active function of the transferred lymph node cells in the formation of the antibody which appears subsequently in the sera of recipient animals, since the prior injection of donor leucocytes, which presumably leads to a specific alteration in the host tissue environment of the transferred cells, affects the appearance of such antibody.

Summary. As observed in previous studies the transfer to irradiated recipient rabbits of donor lymph node cells incubated *in vitro* with soluble antigenic material derived from *Shigella paradysenteriae* was followed by the appearance of agglutinins to these bacilli in the sera of the recipients. If, 4-6 days prior to cell transfer, the recipients were injected intradermally with leucocytes obtained from blood or from peritoneal exudates of the donors, agglutinins failed to appear in the recipients' sera, or appeared in low titer. This effect could be brought about consistently with the pre-injection of 10^6 to 100 x 10^6

leucocytes; not by the pre-injection of rabbit erythrocytes or of leucocytes of horse, cow and chicken blood; and occasionally by the pre-injection of human blood leucocytes.

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Comparative Absorption of Vit. B_{12} Analogues by Normal Humans. III. 5,6-Dichlorobenzimidazole, 5,6-Desdimethylbenzimidazole and 5-Hydroxybenzimidazole Analogues. (23108)

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Preceding communications(1,2) have reported oral absorption of several non-cyanocobalamins to be inferior to cyanocobalamin. Furthermore, all cobalamins were equally effective, upon injection, in inducing excretion of orally administered cobalt-60 labeled forms. These findings indicate the importance of the cyanide group for cobalamin absorption, but offer no suggestion as to influence of structural modifications on oral absorption. It is known that pseudo-vit. B_{12} and its 2-methyl derivative(3), Factor III (3) and the desdimethyl analogue(3,4) of cyanocobalamin exhibit inferior growth activity in chicks. However, direct absorption tests of substituted benzimidazole derivatives in man remain to be performed. Accordingly, urinary excretion tests have been performed with 3 analogues of vit. B_{12} with altered substituents in the benzimidazole moiety. These compounds are (1) the 5,6-dichlorobenzimidazole analogue (DCBA)(5), (2) the 5,6-desdimethylbenzimidazole analogue (DDMBA)(5,6) and (3) 5-hydroxybenzimidazole analogue (Bernhauer's Factor III)(7).

Materials. Cobalt-60 labeled analogues (specific activity $\simeq$ 180 μc/mg) for oral administration were prepared by fermentation in medium containing cobalt-60 and appropriate precursors. Products were isolated by methods previously described for vit. B_{12}, followed by chromatography on cellulose(8).* The DCBA was prepared in the Merck Sharp & Dohme Research Laboratories.†

Method. Urinary excretion method of Schilling(9) was employed as index of absorption. This was(1,2) valid for cobalamins by comparison with 3 other test methods. An oral dose of 2 μg (except for 4 subjects as shown in Table I) of labeled derivative was fed in aqueous solution to normal young adult males, each receiving 1 mg injection (except where otherwise stated) of unlabeled derivative 2 hours after the oral dose. Urine was collected for 24 hours, and, in Study 6, for

* Non-isotopic modifications of DDMBA and Factor III were kindly supplied by Dr. E. L. Smith, Glaxo Laboratories, and by Prof. K. Bernhauer, Aschaffenburger Zellstoffwerke, respectively.

† Preparation of this compound by Drs. F. M. Robinson and I. M. Miller is gratefully acknowledged.

TABLE I. Comparative Urinary Excretion of Structural Analogues of Vit. B_{12} (24 Hr Responses).

Oral admin. Analogue	Dose, μg	Inj. analogue	mg	Study	No. subjects	Avg % of dose excreted ± S.D.
DCBA-Co^{60}	1.4	Cyanocobalamin	1	5*	2	3.9 ± .9
	2	*Idem*		3	4	3.7 ± 1.7
				6	3	3.4 ± .6
		DCBA	1	4	5	6.7 ± 1.4
				6	3	5.3 ± .5
		DDMBA	1	6	3	2.31 ± 1.72
DDMBA-Co^{60}	2	Cyanocobalamin	.2	4	3	.81 ± .11
		"	1	1	4	2.08 ± .91
				2	3	.74 ± .18
		DDMBA	1	2	3	.99 ± .60
				4	5	1.32 ± .63
				6	3	.95 ± .17
		DCBA	1	4	3	1.28 ± .04
		Factor III	1	4	3	1.06 ± .51
Factor III-Co^{60}	1	Cyanocobalamin	1	5*	2	≃ .7†
	2	Factor III	1	4	5	.30 ± .13

* Performed by Dr. L. R. Wasserman, Mt. Sinai Hospital. Personal communication.

† Actual avg is ≃ 1.2% of 1 μg dose. For 2 μg oral dose, response would have been ≃ .7% as recorded for group avg.

second and third days to obviate suspicion of slow elimination. In the latter instances, 1 mg injections of flushing analogues were administered daily to maximize excretion response. Radioactivity measurements have been described elsewhere(1,2). Urinary radioactivity was always calculated as percent of 2 μg dose. The relative effects of injecting the homologous unlabeled analogue and cyanocobalamin on urinary excretion of radioactivity was investigated with all 3 labeled materials. Additional analogues were injected after oral administration of labeled dichloro and desdimethyl derivatives.

Results. Table I lists the results of these excretion studies based upon 24 hours urinary excretion. Six studies, each performed at a different time, are reported. Group average results in terms of "% of Dose Excreted ± Stand. Dev." are reported for each study of each analogue, together with the number of subjects involved. Results of the extension of Study 6 to the second and third days are not tabulated.

Discussion. Results of Study 6 demonstrate that slow elimination of absorbed analogues is not a factor, since one- and 3-day values differ but little. Three-day excretions of DCBA-Co^{60} were 3.7%, 6.0% and 2.5% after injections of cyanocobalamin, DCBA and DDMBA, respectively; and the DDMBA-Co^{60} response was only 1% after flushing with DDMBA. The third day urine contributed <0.05%. For oral cyanocobalamin plus cyanocobalamin injection (*i.e.* standard test), the 1- and 3-day values would have totaled ≃ 12% and ≃ 17% of dose respectively(10,11).

It is evident from the 24-hr. responses (Table I) that none of the 3 substances tested attains the extent of absorption typifying cyanocobalamin. The most effective absorption indication obtained, *i.e.* average excretion of 6.2% ± 1.3% (stand. dev.) of oral dose of DCBA-Co^{60} after injection of 1 mg DCBA, was only half that characterizing cyanocobalamin; and still lower excretions of 3.6% (± 1.3%) and of 2.3% (± 1.7%) were noted after injections of cyanocobalamin‡ and DDMBA respectively.

Absorption indices for DDMBA were exceedingly low, over-all average responses amounting to 1.13% (± 0.56%) for DDMBA injection, 1.30% (± 0.86%) for

‡ Study 5 values are not included in these averages since the oral dose was other than 2 μg.

cyanocobalamin injection, and 1.28% (± 0.04%) and 1.06% (± 0.51%) for respective injections of DCBA and Factor III.

Factor III seemed the least readily absorbed of the 3 derivatives under investigation. The negligible value of 0.30% (± 0.13%) is indistinguishable from the average 0.5% figure reported for cyanocobalamin in pernicious anemia(12). The lower absorption observed of Factor III and DDMBA by normal humans accords with the behavior of these substances when fed to animals. Coates *et al.*(3) found growth stimulating effect of Factor III and DDMBA when fed to chicks to be only 4% and 25-27% respectively of that observed with cyanocobalamin; and a similar inferiority of DDMBA (9% as active as cyanocobalamin) in chicks was reported by Briggs and Fox(4).

The cobalamins and the structural analogues appear to differ in one additional respect. Whereas the extent of induced urinary excretion of the former was independent of cobalamin injected, excretion of DCBA indicates a dependence on the nature of the injected analogue. Unfortunately, this dependence could be extended neither to DDMBA nor to Factor III because of extremely low order of these responses.

Of the several cobalamins and structural analogues of vit. B_{12} studied, cyanocobalamin is most readily absorbed by humans, probably indicating involvement of the cyano-group in the mechanism of oral absorption process. Clearly, however, other factors likewise enter, since all 3 analogues are cyanide complexes. Of these, DCBA, with its 2 symmetrically distributed chlorine atoms, evinces the greatest absorption tendency. One is, accordingly, tempted to suggest that the 5,6 disubstitution is a favorable configuration, and that the difference between DCBA and cyanocobalamin lies in preference of the human organism for methyl rather than chloro groups.

Summary. 1. Urinary excretion tests of oral absorption were performed in normal humans with Co^{60}-labeled 5,6-dichlorobenzimidazole, 5,6-desdimethylbenzimidazole and 5-hydroxybenzimidazole analogues of vitamin B_{12}. 2. Excretion responses obtained with desdimethyl- and hydroxybenzimidazole derivatives are exceedingly low, indicating but slight oral absorption. 3. Behavior of labeled dichlorobenzimidazole analogue indicates good absorption, approximating one-half that of cyanocobalamin. In this case, maximal excretion is attained after flushing injections with unlabeled homologous compound.

The authors wish to acknowledge assistance of the following in preparation of the labeled analogues: Messrs. R. B. Babson and W. O. Brenneman, Merck & Co., for conducting the fermentations and effecting initial purification; Miss Aino Lusi, Mr. David T. Woodbury and Mr. J. P. Gilbert, for final purification. Grants-in-aid (to Dr. Bacon F. Chow) by U. S. Atomic Energy Commission and by Merck & Co., are also acknowledged.

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Effect of Carbutamide on Serum Inorganic Phosphate. (23109)

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Study of the group of hypoglycemia-producing benzene sulfonamide compounds began with the work of Janbon *et al.*(1), and Loubatieres(2). Most investigations were carried out with p-amino-benzene-sulfonamide-isopropyl-thiodiazole (P.A.S.I.T., RP 2254). Interest in the field was enhanced after studies of German workers on N_1-sulfanylil-N_2-n-butylcarbamide (BZ-55, carbutamide)(3,4,5), etc. and the introduction of 1-butyl-3-p-tolyl-sulfonylurea (D-860, tolbutamide)(6,7,8).

Insulin produces a decrease in serum inorganic phosphate in normal and diabetic subjects(9,10,11). In this note, the action of carbutamide on this blood constituent in normal dogs is reported. While this paper was being prepared for publication, contradictory reports appeared. Renold *et al.*(12), mentioned that there is no change in serum inorganic phosphate or urinary phosphate excretion in human subjects after administration of carbutamide or tolbutamide, and Goetz *et al.* (13) reported that tolbutamide produces a decrease in serum inorganic phosphate.

Methods. Six apparently normal dogs weighing between 13.5 and 17.5 kilos were used for the carbutamide experiments. They were fed *ad libitum* on Purina Chow and twice weekly with meat; all foods were withdrawn 14 to 16 hours before experiments were performed. The animals were previously trained with repeated venipunctures to avoid fear and anger reactions. A blood sample was taken and an injection by vein of the sodium salt of carbutamide (20% solution) using 0.25 g/kg body weight was given within one minute. Venous blood samples were then withdrawn at 15′, 30′, 45′, 60′ and 120′. Blood samples were used for duplicate determinations of blood sugar(14) and serum inorganic phosphate(15). In different dogs, 6 experiments with glucose (0.5 g/kg) in 50% solution by vein, and 6 experiments with glucagon free insulin (0.1 U/kg by vein) were carried out for comparative purposes. Blood samples were taken up to 60′. All results are expressed in terms of differences from initial values, in mg/100 ml (Deltas).

Results. The results are shown in Table I and Fig. 1.

Discussion. In our experiments on normal dogs, carbutamide (sodium salt) injected by vein induces a decrease in blood sugar. This change in blood sugar is not accompanied by a significant decrease in serum inorganic phosphate. Insulin produces a marked lowering of serum phosphate, and glucose will also lower this blood constituent. The effect of glucose does not take place in the pancreatectomized animal(16), and seems to be due to an insulin discharge(17) determined by pancreatic stimulation. Since carbutamide shows a very definite hypoglycemic action without exerting any effect on serum inorganic phosphate, this suggests that its action is not mediated through extra insulin secretion. However, since the decrease in blood

TABLE I. Averages and Standard Errors of Initial Values and Deltas of Blood Sugar and Serum Inorganic Phosphate after Administration of Carbutamide, Insulin or Glucose. 6 animals/series.

	0′	15′	30′	45′	60′	120′
	Blood sugar, mg/100 ml					
Carbutamide	72 ± 2	− 9 ± 2	−19 ± 2	−25 ± 1	−29 ± 2	−31 ± 3
Insulin	74 ± 3	−31 ± 3	−35 ± 4	−32 ± 6	−20 ± 4	
Glucose	78 ± 3	+63 ± 14	+17 ± 9	− 3 ± 4	− 9 ± 2	
	Serum inorganic phosphate, mg/100 ml					
Carbutamide	3.80 ± .15	−.07 ± .03	−.08 ± .02	−.07 ± .01	−.08 ± .02	−.10 ± .02
Insulin	3.93 ± .23	−.40 ± .07	−.76 ± .16	−.86 ± .15	−.66 ± .25	
Glucose	4.86 ± .45	−.17 ± .10	−.49 ± .11	−.41 ± .11	−.14 ± .15	

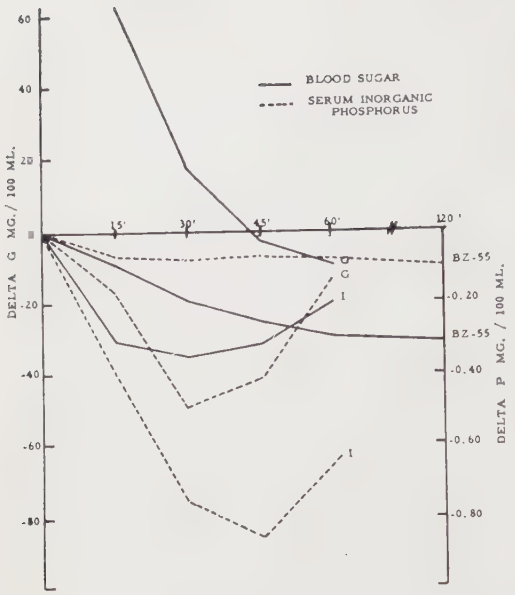

FIG. 1.

sugar induced by carbutamide is gradual, it may be that this time will allow for correction by homeostatic mechanisms, of decrease of serum phosphate, which will thus become masked. Another alternative is that the drug may simultaneously affect the phosphorylation processes.

Summary. The normal trained dog, in fasting conditions, injected by vein with the sodium salt of carbutamide shows a definite decrease of blood sugar, but not significant changes in serum inorganic phosphate. Insulin and glucose produce a definite lowering of serum inorganic phosphate. It seems then, that carbutamide may not lead to a discharge of insulin by the pancreas. However, since the effect of carbutamide on blood sugar is gradual, it may be that its possible action on serum phosphate is masked by other mechanisms.

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Metal Requirements of Alkaline Phosphatases of Human and Rabbit Leukocytes. (23110)

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(Introduced by G. L. Hobby)
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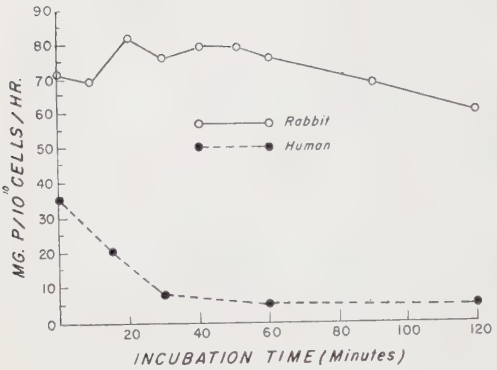
Histochemical and biochemical studies have clearly shown that leukocytes of both man and rabbit are rich in alkaline phosphomonoesterase. Haight and Rossiter(1) showed that the rabbit leukocyte was richer in alkaline phosphatase than the human leukocyte and that magnesium was apparently necessary for complete activation of the enzyme. Valentine and Beck(2) applying the technics of Haight and Rossiter studied alkaline phosphatase activity of the human leukocyte in health and disease. They were able to demonstrate large increases in enzyme content of the leukocyte in infection, polycythemia vera, and in leukemoid states, while sharp decreases were seen in leukemias. In preliminary incubation studies undertaken as part of an investigation into the mechanism of this disease-induced enzymatic change of the leukocyte, striking inhibition of alkaline phosphatase activity became manifest when di-Na ethylenediamine tetraacetate (EDTA) was used. The effects of this chelating agent on leukocyte alkaline phosphatase and results obtained on metal requirements of the enzyme in man and rabbit will be presented. The data indicate the presence of at least 2 alkaline phosphatases in the leukocyte.

Materials and methods. Leukocytes. Fresh human blood was obtained by venipuncture using siliconized syringes. 10 ml of whole blood was added to 1 ml of several anticoagulants. To each 10 ml of blood 5 ml of 2.4% (w/v) bovine fibrinogen in 5% (w/v) polyvinylpyrrolidone (PVP) was added and blood mixed by inversion. The leukocyte-rich supernatant was removed periodically as rapidly as sedimentation of red cells allowed. The leukocytes were transferred to siliconized tubes and were then centrifuged at 500 x G for 5 minutes and the clear supernatant discarded. Cells were washed 3 x with 0.9% saline and resuspended in a measured quantity of the same medium for WBC, RBC, and total polymorphonuclear cell counts. This procedure resulted in average leukocyte recovery of about 75% with a red cell contamination of 2:1. Fresh rabbit blood was obtained by cardiac puncture using siliconized syringes and treated in the same manner. Sedimentation was generally slower than for human blood and leukocyte yield lower. *Anticoagulants.* EDTA (1.5% in 0.7% saline), heparin (1 mg), or sodium citrate (3.8%) as applicable. *Incubation studies.* The packed cells were resuspended in either 1 ml of 0.9% saline or 1 ml of 0.3% EDTA in 0.9% saline and incubated for varying intervals at 37°C. After incubation, all cells were washed 3 x with 0.9% saline prior to assay for phosphatase activity. *Phosphatase determination.* Assays for alkaline phosphatase activity were carried out as described by Valentine(2); the only modifications being in amount and type of heavy metal employed. The general plan of study was to investigate (1) the effects of EDTA on alkaline phosphatase activity of both human and rabbit leukocytes, and (2) to determine the metal requirements of the EDTA-inhibited enzyme.

Results. Effects of incubation of isolated leukocytes with EDTA. Leukocytes of pooled human and of pooled rabbit blood containing EDTA as anticoagulant, were isolated and incubated with EDTA. The cells were then assayed at varying intervals for alkaline phosphatase activity with no metal added. Fig. 1 shows the difference in behavior between rabbit and human leukocytes. The initial activity of pooled normal rabbit leukocytes was higher than that of pooled normal human leukocytes. Further, the alkaline phosphatase activity of the rabbit leukocyte was not appreciably inhibited during a 2 hr incubation period, whereas the human cell showed a rapid decrease so that in 30 min. approxi-



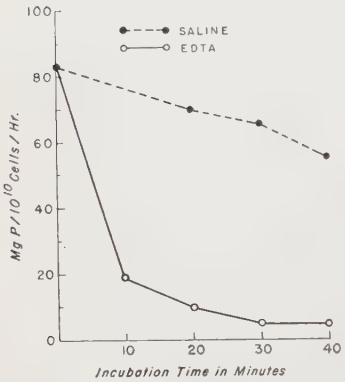


FIG. 1 (top). Effect of EDTA on alkaline phosphatase of isolated human and rabbit leukocytes (anticoagulant: EDTA).

FIG. 2 (bottom). Effect of EDTA on alkaline phosphatase of isolated human leukocytes (anticoagulant: heparin).

mately 80% of initial activity had been lost. After 60 min. no further inhibition was seen.

To determine whether the demonstrated inhibition of alkaline phosphatase of the human cell was due solely to the effects of EDTA, human leukocytes were isolated from heparinized blood. Aliquots were incubated in EDTA and in 0.9% saline and the phosphatase activity compared at intervals. Fig. 2 again demonstrates striking inhibitory action of EDTA. However, the control cells incubated in saline also showed a progressive, though considerably smaller, decrease in enzyme activity. There was no significant decrease in cell count throughout the period of study. In view of the chelating properties of EDTA, an attempt was made to reactivate alkaline phosphatase activity by addition of known metal activators. The addition of magnesium in final concentration of 2.5×10^{-3}M in the phosphatase assay had no effect on level of activity. Upon addition of zinc in final concentration of 2.5×10^{-4}M however, phosphatase activity was restored to original levels in both cases.

Effects of magnesium and zinc on leukocyte alkaline phosphatase activity. The results of a study on effect of varying concentrations of zinc and magnesium on EDTA exposed human and rabbit leukocytes are shown in Table I. The rabbit cell showed a high alkaline phosphatase activity despite 1 hr incubation in EDTA. Addition of magnesium in various concentrations up to 5×10^{-2}M produced some decrease in alkaline phosphatase activity. Similarly, final concentrations of zinc at 2.5×10^{-4}M produced a sharp drop in activity. These inhibitory effects have been attributed(3) to metal substrate complexes formed at high metal concentrations. The EDTA-inhibited human leukocyte, on the other hand, showed a slight rise with increasing concentration of magnesium. However, addition of 5×10^{-5}M zinc caused approximately a 50-fold increase in alkaline phosphatase activity. Here too a drop in activ-

TABLE I. Effect of Zinc and Magnesium on Human and Rabbit Leucocytes Incubated for 1 Hr at 37°C with EDTA.

Magnesium			Zinc		
	mg P/10^{10} cells/hr			mg P/10^{10} cells/hr	
Final molarity	Human*	Rabbit†	Final molarity	Human*	Rabbit†
0	2.4	70.4	0	2.4	70.4
2.5×10^{-4}	2.8	69.2	5×10^{-6}	2.8	64.0
$5 \times$ "	2.0	64.0	1.25×10^{-5}	71.2	69.2
2.5×10^{-3}	3.6	60.0	$2.5 \times$ "	100.0	77.2
$5 \times$ "	4.8	57.2	$5 \times$ "	121.0	81.2
2.5×10^{-2}	4.0	47.0	2.5×10^{-4}	39.0	15.6
$5 \times$ "	4.0	53.2			

* Pooled polycythemic + normal.

† Pooled normal.

TABLE II. Effect of Magnesium and Zinc on the Alkaline Phosphatase Activity of Normal Human Leukocytes.

Anti-coagulant	No. of patients	Final molarity metal	Mean activity, mg P/10^{10} cells/hr	Range
EDTA	39	None	3.8 ± 2.1	0.8- 7.8
"	46	2.5×10^{-3} Mg	8.6 ± 5.4	0 -26.4
"	25	5.0×10^{-5} Zn	16.7 ±11.0	2.4-44.8
"	25	2.5×10^{-4} Zn	1.5 ± 1.6	0 - 6.0
Citrate	23	None	25.5 ±15.1	8.0-61.2
"	18	2.5×10^{-3} Mg	31.2 ±16.6	4.8-56.4
"	22	5.0×10^{-5} Zn	23.2 ±11.2	7.6-44.4

ity at high zinc concentration was seen. Loss in phosphatase activity of human leukocytes upon incubation with saline seen in Fig. 2 may possibly be attributed to leakage of zinc from the cell during incubation period, and in the wash periods after incubation.

The addition of EDTA to whole blood produced a progressive decline in alkaline phosphatase activity of leukocytes. After 90 min. at 25°C, there was a 65% decrease in activity of cells subsequently isolated. This activity could be restored by the addition of zinc in final concentration of 5 x 10^{-5}M.

Normal values. Normal values for leukocyte alkaline phosphatase have been determined in man using cells isolated from citrated blood and compared with those obtained with and without the subsequent addition of metals. Table II shows that in man the highest alkaline phosphatase activity can be obtained by using cells isolated from citrated blood and that addition of either magnesium or zinc to this system contributed little. On the other hand, cells isolated from EDTA treated blood required zinc for activation; magnesium produced little response. The maximum activation of EDTA treated cells with zinc or magnesium does not reach the level obtained with cells isolated from citrated blood and this may be due to the fact that we are working at sub-optimal zinc concentrations, or possibly sub-optimal levels of other activators; *e.g.* the alpha-amino acids.

Discussion. The phosphatases require an alpha-amino acid as well as a bivalent metallic ion for their activation(4). There have, however, been many contradictory reports concerning the role of metal ions in activation of alkaline phosphatases. In addition, the question as to number and identity of different phosphatases has still not been resolved. Cloeten(5-7) presented evidence that alkaline phosphatases are metal complexes and concluded from dialysis and inhibition studies that both magnesium and zinc were involved in these complexes. He argued that the alkaline phosphatases of different organs were not identical, probably being a mixture of 2 enzymes. Further, activity of the enzyme extracted from any given tissue was determined by concentrations of these 2 alkaline phosphatases which varied from organ to organ. More recently, Gryder, Friedenwald, and Carlson(3) working with rat kidney reported on the presence of 2 alkaline phosphatases which they called phosphatases A and B. Phosphatase A required both glycine and zinc for activation while phosphatase B required magnesium only and was inhibited by calcium and zinc. The data here presented suggest that there are also 2 alkaline phosphatases in the human leukocyte, one requiring magnesium and the other zinc for activation. In this regard, the rise in leukocyte alkaline phosphatase obtained by Wiltshaw and Moloney(8) following incubation of human leukocytes (from EDTA blood) with serum can probably be attributed to the zinc content of the serum (5 x 10^{-4}M(9)), rather than to some "factor in the leukocyte." Our own studies confirm the activating properties of serum on the EDTA inhibited leukocyte alkaline phosphatase.

Of interest is the fact that Vallee(9) has found a high zinc concentration in the human leukocyte and there seems to be a gross parallel between alkaline phosphatase activity and zinc content of the cell in disease states. The relationship, if any, existing between these 2 cellular constituents is not known. Preliminary results of studies now being carried out suggest that elevation of alkaline phosphatase activity seen in the leukemoid states and in infection in man is due primarily to elevation of the zinc activated phosphatase rather than the magnesium activated enzyme.

In the rabbit, the situation is grossly different inasmuch as EDTA shows no appreci-

able effect on leukocyte alkaline phosphatase activity and zinc or magnesium have no activating effect. Obviously, there is a species difference either in the leukocyte or in the kind of phosphatase present. By histochemical studies we have been able to show a marked increase in the leukocyte alkaline phosphatase of the rabbit following injection of vaccines, pyrogens, etc., but the enzyme type has not as yet been characterized.

Freiman(10) has carried out rather comprehensive histochemical studies of inhibition of alkaline phosphatase activity of various tissues by EDTA and concluded that a number of alkaline phosphatases exist. Our findings indicate that even a brief incubation of human leukocytes with EDTA results in a decrease in alkaline phosphatase activity. EDTA currently enjoys much popularity as an anticoagulant for blood and is widely used in tissue decalcification. Obviously the chelating properties of EDTA for ions other than calcium must be kept in mind in any investigation of alkaline phosphatase activity in blood or tissues.

Summary. EDTA inhibits alkaline phosphatase activity of the human leukocyte. The rabbit leukocyte is, however, not so affected. There seem to be 2 alkaline phosphatases in the human leukocytes: one requiring magnesium and the other zinc for its activation.

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Spleen DPNH Cytochrome *c* Reductase Activity In X-Irradiated Rats.* (23111)

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With either α-ketoglutarate or succinate as substrate, several laboratories(1-3) have reported a decrease in oxidative phosphorylation by mitochondria and homogenates prepared from spleens of rats and mice exposed to lethal doses of X-irradiation. Oxygen consumption was also found to be diminished in the presence of either α-ketoglutarate(2,3) or malate(4). Since phosphorylation is coupled to DPNH oxidation in the sequence DPNH → cytochrome *c*, it was considered of interest to determine whether interference with DPNH cytochrome *c* reductase activity in the spleens of X-rayed animals was involved in the lowered oxidation rates or linked in some way to the diminished P:O ratios. Another area on which some light might be shed by the study of DPNH cytochrome *c* reductase activity in irradiated animals is in the relation of cell structure to function. The presence of a high concentration of this enzyme in liver(5) and spleen† microsomes is somewhat of an oddity, since most of the known linked respiratory systems are associated with the mitochondria. Loss of microsomal DPNH cytochrome *c* reductase to the exclusion of the mitochondrial enzyme would appear to cast

* Supported by contract with Division of Biology and Medicine, U. S. Atomic Energy Commission.

The following abbreviation is employed: DPNH = reduced diphosphopyridine nucleotide.

† Eichel, H. J., *J. Biophys. and Biochem. Cytology,* 1957, v3, no. 3, in press.

the latter in a more significant physiological role.

. The effect of 700 r of whole-body X-irradiation on the DPNH cytochrome *c* reductase activity of rat spleen homogenates, and subcellular fractions isolated from them, is described here.

Methods. A. Irradiation procedure and experimental design. Two series of experiments were performed with male rats obtained from the Wistar Institute. In the first series at the time of irradiation, the animals weighed from 110 to 150 g, and in the second, from 130 to 155 g. The rats were given, in a single exposure, a total dose of 700 r of 220 KV X-rays filtered through 0.25 mm Cu and 1 mm Al at an average rate of 87.1 r/minute and a target distance of 50 cm. The animals were irradiated in groups of 6 in metabolism cages. Food was withheld from exposed and control rats for the first 20 to 22 hours after irradiation. Purina dog chow checkers were then fed to both groups, but to correct for any changes in enzyme activity that might be caused by the decreased food intake of the irradiated rats. the control animals were carefully pair-fed against the exposed. Water was provided *ad libitum*. In the first series, rats were killed by a blow on the head, and in the second, by ether anesthesia. Six control and 6 experimental rats were sacrificed on the first and fourth days after irradiation in the first experiment. In the second experiment, 6 rats from each group were sacrificed on the first, second, and fourth days after exposure. Owing to the relatively long time required to complete each fractionation, it was deemed advisable to pool the spleens of 3 animals for a single fractionation. Hence, on all but 1 day, 4 homogenates and 4 complete fractionations were made, 2 each with spleens grouped from 3 X-rayed rats and 2 each with organs from 3 control animals. On day 4 of the first experiment, only homogenates were prepared. The first, second, and fourth days after irradiation actually correspond to 13, 37, and 85 hours post-irradiation. *B. Preparation and fractionation of spleen homogenates.* After excision, spleens were washed free of adhering blood and homogenized in cold 0.25 M sucrose with a Ten Broeck glass homogenizer. Each homogenate (3 spleens) was diluted to 10 ml with sucrose, 2 ml were removed for nitrogen analysis, and the remainder was fractionated, with some changes, according to the method of Hogeboom *et al.*(6) as modified by Schneider and Hogeboom(7). All operations were carried out at 0–4° C. The nuclear fraction was collected by centrifugation at 600 X g for 10 minutes, the mitochondria by centrifugation at 5,000 X g for 15 minutes, and the microsomes and "fluffy layer" by centrifugation at 110,000 X g for 60 minutes. The nuclear and mitochondrial fractions were each washed once with sucrose. Four such fractionation procedures, performed simultaneously, required about 6 hours to complete. A study of the intracellular localization of DPNH cytochrome *c* reductase and cytochrome *c* oxidase in fractions derived from normal rat spleen homogenates has been reported. A detailed account of this and related studies will be published elsewhere.† The recovery of most of the deoxypentosenucleic acid in the 600 X g fraction (nuclear) and 45% of the ribonucleic acid in the 110,000 X g fraction (microsomes), the high specific and total activities of succinic dehydrogenase and cytochrome *c* oxidase in the 5,000 X g fraction (mitochondria), and the low specific and total activities of these enzymes in the 110,000 X g fraction provide strong chemical and enzymatic evidence that the isolated fractions studied here are analogous to those which have been separated from liver homogenates at similar centrifugal forces. The distribution pattern of DPNH cytochrome *c* reductase activity in the particulate fractions of spleen homogenates is similar to that observed by Hogeboom for liver(5) except that, per mg of nitrogen, the enzyme is concentrated to a much greater degree in spleen mitochondria. This is probably a reflection of the fact that the mitochondrial fraction represents only about 5-8% of the total nitrogen content of spleen homogenates. *C. Enzyme assay, materials.* DPNH cytochrome *c* reductase activity was determined spectrophotometrically (Beckman DU) at 25°C essentially according to the method of Potter and Albaum(8). The following components were added to each cuvette in the

order given: 0.025 to 0.2 ml of a suitable dilution of homogenate or fraction, 0.2 ml of 0.03 M KCN, 0.3 ml of 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4, water to make a volume of 3 ml, 1.43 mg of cytochrome *c* in 0.25 to 0.75 ml of water, and 0.3 to 0.4 micromole of DPNH in 0.7 ml of 0.1 M KH_2PO_4–K_2HPO_4, pH 7.4. The omission of nicotinamide did not affect reductase activity. Zero time was taken as time of mixing (by inversion) of the covered cuvette immediately after addition of DPNH. The first reading, at 550 mμ, was the time required for the instrument to reach a predetermined density value. Subsequent readings were made at densities 0.010 or 0.020 above the preceding ones until 6 to 10 readings were taken, or, in the case of the supernatant fraction, 2 to 3 minutes had elapsed. Activity of each fraction (with exception of the supernatant) was reasonably proportional to the amount of tissue present. The frequent absence of proportionality between activity of the supernatant fraction and the volume tested made accurate calculations of the absolute activity of this fraction difficult. The reason for this phenomenon is not known. The specific activity of homogenates and all fractions was calculated with the value 1.96 $x10^7$ sq cm/mole as the difference between molecular extinction coefficients of oxidized and reduced cytochrome *c*. The reaction rates were zero order with respect to cytochrome *c* concentration, and specific activity was expressed as *micromoles of cytochrome c reduced/minute/mg of nitrogen* (of each fraction added to the cuvette). Total activity (micromoles of cytochrome *c* reduced/minute) was obtained by multiplying the specific activity of each homogenate or fraction by its respective nitrogen content. Nitrogen was determined by a micro-Kjeldahl procedure. The DPNH was prepared chemically with sodium hydrosulfite(9) from DPN (Sigma Chemical Co.) which was 80% pure by alcohol dehydrogenase assay(10). Beef heart cytochrome *c* of about 70% purity, based on extinction coefficients of oxidized and reduced cytochrome *c*, was obtained from Sigma.

Results. The distribution of nitrogen in the subcellular fractions isolated from spleen homogenates of control and X-rayed rats is presented in Table I. Due to splenic atrophy following X-irradiation, it can be seen that in each experiment on the first day post-irradiation, spleens of X-rayed animals contained 25 to 35% less nitrogen than did those of controls. In Exp. No. 2, day 2, the nitrogen content of control spleens was essentially unchanged while that of the X-rayed organs diminished to an even greater degree. By day 4, in each experiment, the nitrogen content of X-rayed spleens was still further reduced while that of the control spleens had fallen by 40% as compared to day 1 in Exp. No. 1 and by 60% as compared to days 1 and 2 in Exp. No. 2. In each experiment, data for average body weights of control animals for day 4 suggest that the decreased nitrogen content (and wet weight) of the spleens was probably a result of acute inanition imposed by pair-feeding. In Exp. No. 2, the average body weights (g) of the 6 X-rayed rats studied on each of days 1, 2, and 4 were 137, 127, and 110, respectively, and of the 6 control rats, 144, 135, and 110, respectively. In Exp. No. 1, body weights of X-rayed animals averaged 139 and 127 g on days 1 and 4, respectively, while those of the control rats averaged 124 and 116 g, respectively.

The data of Table I also show that the % recovery of homogenate nitrogen in spleen mitochondria from irradiated rats was twice as high as from control animals. This was also observed by Maxwell and Ashwell in spleen mitochondria obtained from X-irradiated mice(1). They concluded that some of the tissue debris produced by irradiation sediments as inert nitrogenous material with the mitochondria. The % recovery of nitrogen in spleen microsomes and supernatant from irradiated rats was also slightly higher than from control animals. On the other hand, more nitrogen was recovered in the nuclear fractions of spleens from control rats than from exposed animals. It is worthwhile noting that the % recoveries of nitrogen in mitochondrial fractions of control spleens listed in Table I were less than those (8%) found in earlier experiments.† The explanation for this difference is not known, although in the earlier work, the rats were fed *ad libi-*

TABLE I. Distribution of Nitrogen in Subcellular Fractions Isolated from Spleens of Control and Irradiated Rats. Each value represents avg of 3 pooled spleens.

			Total nitrogen (mg)				Recovery (%)			
			Irradiated		Control		Irradiated		Control	
Exp. No.	Day	Fraction	1	2	1	2	1	2	1	2
1	1	H*	25.6	28.3	36.2	34.4	100	100	100	100
		N	11.7	12.8	20.1	19.5	45.7	45.3	55.5	56.5
		M	2.5	2.8	1.8	1.7	9.7	10.0	4.9	4.9
		P	2.7	3.2	3.5	3.5	10.6	11.3	9.6	10.2
		S	7.7	8.4	9.3	8.8	30.2	29.8	25.6	25.5
		Recovery	24.6	27.2	34.7	33.5	96.2	96.4	95.6	97.1
	4	H	12.5	12.3	23.3	16.7				
2	1	H	24.8	22.4	36.1	36.7	100	100	100	100
		N	11.9	9.8	21.2	21.9	48.0	43.8	58.8	59.6
		M	2.0	1.9	1.7	1.7	8.2	8.5	4.7	4.6
		P	3.1	2.8	3.5	3.5	12.5	12.3	9.6	9.5
		S	6.8	6.7	8.6	8.6	27.6	29.8	23.7	23.3
		Recovery	23.8	21.2	35.0	35.7	96.3	94.4	96.8	97.0
	2	H	16.1	17.8	41.0	38.8	100	100	100	100
		N	6.3	7.8	23.9	22.9	39.0	44.0	58.4	60.0
		M	1.5	1.5	1.8	1.8	9.3	8.2	4.4	4.6
		P	2.2	2.1	3.9	4.0	13.4	12.0	9.6	10.2
		S	4.9	5.5	10.3	10.5	30.5	31.0	25.1	27.1
		Recovery	14.8	16.9	39.9	39.1	92.2	95.2	97.5	101.9
	4	H	9.2	10.1	16.0	14.6	100	100	100	100
		N	3.8	3.9	7.8	7.1	41.3	38.3	48.9	48.2
		M	1.1	1.0	1.1	1.0	11.4	10.2	7.1	6.6
		P	1.3	1.3	1.9	1.7	13.8	12.9	11.6	11.8
		S	2.5	3.1	3.8	4.0	27.6	30.4	24.0	27.1
		Recovery	8.7	9.3	14.7	13.7	94.1	91.8	91.6	93.7

* H = homogenate, N = nuclei, M = mitochondria, P = microsomes, S = supernatant.

tum.

Distribution of DPNH cytochrome *c* reductase activity in fractions obtained from spleen homogenates of control and irradiated rats is presented in Table II. On the first day following irradiation, specific activities of the homogenates of X-rayed spleens were increased by an average of 43 and 42% in Exp. No. 1 and 2, respectively. On day 2 of Exp. No. 2, the activities of these homogenates were again 42% higher than the control levels. On day 4 of Exp. No. 2, the activities of the 2 control homogenates had risen by 36% over the average control value for day 1. Therefore, while the activities of X-rayed homogenates were still elevated, their average figure for day 4 was only 13% greater than the average control activity for that day. The data of Table II show a similar relationship between the homogenate activities of days 1 and 4 in Exp. No. 1. It is likely that the increased activities of control homogenates on day 4 were a reflection of the loss of general spleen nitrogen due to the severely restricted food intake of the control rats. If non-cytochrome reductase nitrogen were lost more rapidly than reductase nitrogen, the net result would be an increased concentration of enzyme with respect to nitrogen. Activities of control homogenates and their nitrogen content on day 2, Exp. No. 2 are in agreement with this idea. The data in Table I show that on day 2 nitrogen content (essentially proportional to wet weight) of control spleens was unchanged when compared to nitrogen content of control spleens of day 1, and in Table II, little change in enzyme activities of control homogenates of day 2 is noted as compared to values of day 1. Thus, under the experimental conditions described, the interval between days 2 and 4 was critical in effecting a great reduction in nitrogen content of spleens of control rats. The view is favored here that the early increased specific

TABLE II. Distribution of DPNH Cytochrome *c* Reductase Activity in Subcellular Fractions Isolated from Spleens of Control and Irradiated Rats. Each value represents the avg of 3 pooled spleens.

			Specific activity*			
			Irradiated		Control	
Exp. No.	Day	Fraction	1	2	1	2
1	1	H†	.317	.320	.232	.214
		N	.100	.111	.104	.123
		M	.838	.784	1.149	1.230
		P	1.118	.985	.885	.909
		S	.094	.077	.076	.080
	4	H	.392	.402	.340	.366
2	1	H	.364	.419	.248	.304
		N	.110	.125	.141	.139
		M	1.197	1.138	1.638	1.612
		P	.923	.989	.840	.916
		S	.170	.180	.158	.179
	2	H	.435	.388	.288	.292
		N	.145	.116	.133	.118
		M	1.157	1.053	1.691	1.475
		P	1.034	.959	.735	.757
		S	.200	.190	.160	.158
	4	H	.456	.418	.401	.371
		N	.180	.157	.185	.193
		M	1.126	1.056	1.528	1.736
		P	.992	.902	1.123	1.080
		S	.200	.190	.120	.100

* Micromoles of cytochrome *c* reduced/min./mg of nitrogen.

† H = homogenate, N = nuclei, M = mitochondria, P = microsomes, S = supernatant.

activities of the homogenates prepared from spleens of irradiated animals were also due to loss of non-enzyme nitrogen rather than to a true activation of the enzyme. In the case of irradiated rats, the decrease in spleen nitrogen content (35% less than the controls) was apparent by day 1 and was accompanied by a 42% increase in specific activity of DPNH cytochrome *c* reductase. However, it should be noted that between days 1 and 4, a further marked drop in nitrogen content of X-rayed spleens occurred (60% less than the X-rayed levels for day 1), but reductase activity increased by only an additional 12%. This observation might be interpreted to mean that during this interval a considerable amount of enzyme was lost as well as general spleen nitrogen.

Specific activities of the nuclear, microsomal, and supernatant fractions after irradiation showed no striking changes as compared to control values (Table II). However, specific activities of the mitochondrial fractions from spleens of the irradiated animals were decreased by about 30% as compared to control activities. This decrease was probably due, in part, to the fact that the % of whole homogenate nitrogen recovered in mitochondria after irradiation was twice that found in mitochondria derived from control spleens.

In Exp. No. 2, total DPNH cytochrome *c* reductase activity (micromoles of cytochrome *c* reduced/minute) of spleen homogenates from control rats averaged 10.1, 11.6, and 5.9, and that of homogenates from the X-rayed rats averaged 9.2, 7.0, and 4.2 on the first, second, and fourth days after irradiation, respectively. Compared to control levels, this amounted to a loss by the X-rayed spleens of 9, 40, and 30% of the total reductase activity on the 3 days. In Exp. No. 1, by the fourth day, total enzyme activity of X-rayed spleens also averaged 30% less than that of controls, although on the first day it was 9% higher than the control values. On each day, of the total enzyme activity of the spleen homogenates from either the control or irradiated animals, 25 to 30% was recovered in the mitochondria and 30 to 37% in the microsomes. The total reductase activity recovered in the nuclear fractions of X-rayed spleens on each day was only about half that found in the nuclear fractions of control spleens. This might have been due to (1) the somewhat greater nitrogen recovery in the nuclear fractions from control spleen homogenates and (2) the fact that an average of only 72% of the total activity of the original homogenates was recovered among all fractions prepared from irradiated spleens while 88% of the total activity was recovered among the 4 fractions obtained from the control spleens. In a previous study, the % recovery of total DPNH cytochrome *c* reductase activity from normal rat spleen homogenates ranged from 90 to 100 (avg = 103) for 10 separate fractionations.†

The unimpaired DPNH cytochrome *c* reductase activity of homogenates and particulate fractions prepared from spleens of X-irradiated rats is consistent with numerous reports in the literature indicating that the activities of tissue respiratory enzymes (dehydro-

genases and cytochrome *c* oxidase) are little affected by *in vivo* irradiation at lethal levels.

Summary. Rats were subjected to 700 r of whole-body X-irradiation. At intervals of 13 to 85 hours after exposure, homogenates were prepared from spleens of irradiated and control animals and separated into 4 subcellular fractions by differential centrifugation. Per mg of nitrogen, DPNH cytochrome *c* reductase specific activity of the homogenates from X-rayed rats was increased significantly over the control levels at 13 and 37 hours after exposure. At 85 hours, the difference was much less owing to an increased specific activity of the enzyme in the control homogenates, as well. Expressed on the basis of nitrogen, cytochrome reductase specific activities of nuclear, microsomal, and supernatant fractions after irradiation showed no striking changes as compared to control values. Specific activities of the mitochondrial fractions from spleens of irradiated animals were decreased significantly as compared to control levels. The % of total DPNH cytochrome *c* reductase activity recovered in the 4 fractions prepared from the spleens of irradiated and control animals was essentially the same.

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Effects of Adrenergic and Cholinergic Drugs Injected by Intra-Carotid Route on Electrical Activity of Brain. (23112)

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It has previously been reported(Longo, 10) that the EEG pattern provoked by intracarotid injection of acetylcholine (ACh) in rabbits is similar to the "activation" or "arousal" response produced by external stimuli. Similar observations have been made by Rinaldi and Himwich(15), who consider this effect as a specific action of cholinergic drugs on the reticular activating system. On the other hand, much experimental data suggest that cholinergic drugs are not the sole mediators of the activation response. In fact, it has been demonstrated that epinephrine and amphetamine are also able to elicit an activation picture in the EEG of experimental animals(Bonvallet *et al.,* 1; Elkes *et al.,* 6; Schallek and Walz, 17). This paper is concerned with the modifications of EEG after injection of acetylcholine, eserine, epinephrine, and amphetamine directly into the brain circulation.

Methods. Fourteen non-curarized unanesthetized rabbits and 12 "cerveau isolé" preparations (for details of "cerveau isolé" preparation in rabbit, *cf.* Longo and Silvestrini, 12) were used. Blood pressure was measured in majority of experiments, connecting femoral artery to mercury manometer. Some additional experiments were carried out on cats curarized with d-tubocurarine. The technic(Longo, 10) consists in cannulating the internal carotid in the rabbit so that the injected drug reaches the brain directly. Precautions were taken to avoid arousal reactions

caused by external solicitation during the injection, and repeated controls were made injecting saline. Cortical and subcortical EEG were recorded by technics reported elsewhere (Longo, 11). Eserine and amphetamine were injected in the form of salicylate and sulfate salts respectively; acetylcholine, either as hydrochloride or perchlorate salt, epinephrine in the form of bitartrate. All doses are reported as total amount given to animal and are expressed as weight of free bases. At the end of the experiment, an intracarotid injection of methylene blue or of India ink was given for macroscopic control of the area that this route supplies.

Results. Administration of drugs into internal carotid of normal rabbit. Acetylcholine. In all of the animals an arousal response was first elicited by injecting intracarotid ACh. The results obtained previously (Longo, 10) were confirmed. All animals responded to ACh; however, effective doses varied in different animals from 0.5 to 2 μg. The injection produced a desynchronisation of the tracing with very short (1-2 seconds) latent periods. This EEG arousal was generally accompanied by tachypnoea and searching movements of the head, with higher doses (10-20 μg) by efforts to escape. Repeated administration of ACh given at intervals of 10 minutes provoked always the same response in the EEG. The records of the blood pressure showed that no modification followed the small doses; a delayed and short lasting fall was noticed after high doses. The same high dose of ACh, by intravenous injection, produced considerable lowering of blood pressure and flattening of the EEG, but no arousal reaction.

Eserine. The general procedure followed was to administer only one drug in every experiment after sensitivity to ACh was tested. Five animals received eserine. A single dose of 10 μg was never followed by EEG changes. When a total amount of 30 to 50 μg was reached by repeated administration or in a single administration, a distinct activation of the tracing appeared after a latent period of 1-2 minutes and lasted for 30 minutes or more. Attention should be drawn to the fact that external stimulation of the animal immediately after injection of the drug reduces the latent period, as it gives rise to prompt and prolonged desynchronisation. These EEG patterns of arousal were not accompanied by behavioural changes seen after injection of ACh.

Epinephrine. In 4 rabbits injection of doses up to 20 μg caused no modifications of the EEG. When the same amount of drug was administered intravenously, activation of the tracing was rarely obtained; rather, on those occasions when epinephrine produced any change at all, a tendency to increase spindling was noticed. Curarized cats have been employed in a number of experiments; intravenous injection of 10-20 μg of epinephrine provoked in this animal an activation of the EEG. The injection of the drug into the common carotid artery in doses up to 10 μg was not followed by any activation; with larger doses (20-30 μg) activation of the tracing was frequently noted, but this appeared 10-20 seconds later when a rise of arterial blood pressure was observed, due to entrance of epinephrine into the general circulation.

Amphetamine. Five animals received amphetamine. No modification of the electrical tracing was observed up to 100 μg. When a cumulative dose of 200-300 μg was reached, a desynchronisation of the EEG and signs of excitement and tachypnoea were noted.

Administration of drugs into carotid artery of the "cerveau isolé" preparation. At least 1 hour elapsed between the cutting of the brain stem and the beginning of the experiment. After administration of acetylcholine up to 20 μg into the carotid artery, the activation which is obtained in the normal animal was never observed. In 4 out of 10 experiments a change in pattern was noticed, consisting in a discharge of high voltage waves at 8-12 c/sec. (Fig. 1), especially in occipital tracings. A motor reaction and tachypnoea always followed the injection.

The effects of administration of eserine and amphetamine were the same as in the normal animal; in the "cerveau isolé," however, desynchronisation was obtained only with larger doses (0.05-0.1 mg of eserine and 0.5-1 mg of amphetamine). Epinephrine was not

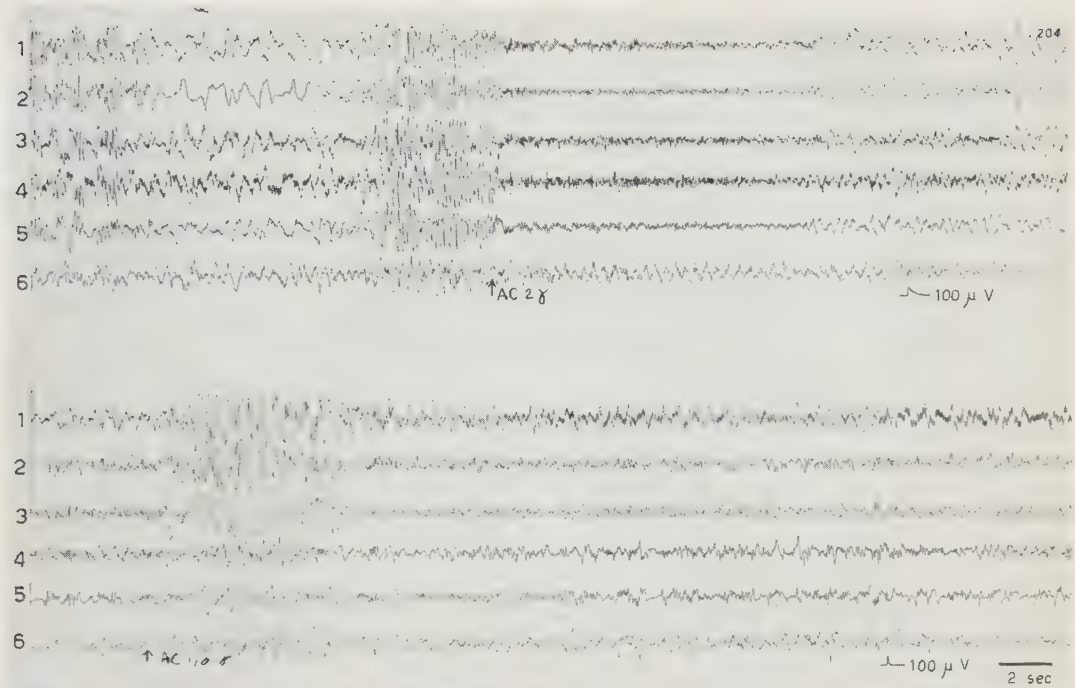

FIG. 1. Action of acetylcholine administered by intracarotid route in normal and "cerveau isolé" rabbit. *First tracing:* Rabbit 2 kg. Cannula in left internal carotid. At arrow: acetylcholine 2 μg. Activation in all leads. Searching movements of head. *Leads:* 1—L. fr.-occ.; 2—R. frontal; 3—L. fr.-R. fr.; 4—L. frontal; 5—R. fr.-occ.; 6—anteromedial thalamus. *Second tracing:* Rabbit 2, 5 kg, "cerveau isolé." Cannula in internal left carotid. At arrow: acetylcholine 5 μg (10 μg of chlorate salt). EEG response is completely different from the above and consists in discharge of high voltage waves. An intense motor reaction is present. For demonstrative purposes, a tracing of "cerveau isolé" with low voltage background was chosen. *Leads:* 1—L. occipital; 2—L. parietal; 3—L. frontal; 4—R. frontal; 5—R. parietal; 6—R. occipital.

tested in the "cerveau isolé" animals.

Discussion. The activating effect of epinephrine injected intravenously, described by Bonvallet *et al.*(1), in the cat was confirmed by the present authors; however, no activation was obtained with smaller doses by intracarotid injection. In the rabbit, the activation of EEG very rarely follows administration of epinephrine, either by intravenous or intracarotid route. DeMaar and Martin found that in the spinal vagotomized cat l-epinephrine produced activation that became increasingly feeble with repetitive doses and that synchronisation and spindling became predominant. Rothballer(16) found that in curarized cats with an intact brain stem epinephrine was not able to produce a marked activation or to completely abolish slow waves and spindling activity. In view of the results obtained in rabbits by ourselves and in cats by the forementioned authors, it appears unlikely that epinephrine plays a major role in the activation of the EEG mediated through the reticular formation.

When injected directly in the cerebral circulation, amphetamine produced an EEG response very similar to the one observed by intravenous administration of the same dose. The fact that amphetamine is still active in the "cerveau isolé" means that its action is relatively independent of the presence of the mesencephalic activating system and can be exerted on higher structures (hypothalamus).

When injected intracarotidly, eserine failed to show an action similar to ACh, *i.e.*, a desynchronisation of EEG, characterized by rapid onset and short duration. Previous experiments(Longo, 10) have shown that the

same rapid and short activation characteristic of ACh could be obtained with other substances (tetramethylammonium iodide, succinylcholine chloride, d-tubocurarine chloride). It is, therefore, suggested that whereas ACh and other substances possessing a quaternary nitrogen produce their effects by means of immediate action, the slower onset of the action of eserine is probably related to certain enzymatic changes. The difference between the effect of eserine and ACh is also evident in the "cerveau isolé" preparation. The activating action of ACh on this preparation, obtained by Rinaldi and Himwich(15), could not be confirmed. On the contrary, it appears possible to reproduce, sometimes, by means of intracarotid injections, the synchronising and convulsant effect demonstrated after topical application of the drug on the cortex(Miller *et al.*, 13; Funderburk and Case, 8). Eserine, on the contrary, is still active in producing activation in the "cerveau isolé". Desmedt and LaGrutta(5) claim that the desynchronisation, due to anticholinesterase drugs, is due to the inhibition of the cholinesterase present in the neuroglia and is not at all connected with functional variations. In this connection, it is worthwhile to remember that eserine never produces any behavioural changes, while ACh always produces searching movements and dyspnoea.

The lack of desynchronisation in the "cerveau isolé" may have some bearing upon the general problem of the locus and mechanism of the activation produced by ACh. A peripheral effect on receptors of the arterial walls or of the dura cannot be excluded. In this case, the lack of desynchronisation in the "cerveau isolé" would be due to deafferentation of the cerebrum.

It is also possible that ACh may influence the vestibular nuclei(Essig *et al.*, 7; Delgado, 4) or the respiratory centers(Gesell *et al.*, 9) or the cerebellar cortex(Crossland and Mitchell, 3) and thus, indirectly through centripetal pathways, provoke generalized activation. In fact, in the "cerveau isolé," ACh still produces motor and respiratory excitement, while the desynchronisation is not induced because of interruption of the rostral connection.

The action of ACh may be exerted directly on the cerebral cortex. In anatomical controls, the cortical layers of the cerebrum and of the cerebellum homolateral to the injected side were found stained. Only on rare occasions were the infundibular area and the dorso-anterior part of the thalamus reached; the dye was found also in the choroid plexus. The mesencephalic brain stem was never directly injected with the dye. However, since a difference in rate of diffusion between the dyes and ACh is known to exist, we must consider the anatomical findings only as orientative information.

It is known (Bremer and Terzuolo, 2) that it is possible to obtain a generalized desynchronisation after electrical stimulation of appropriate regions of the cortex, which is dependent on the cortico-reticular and reticulo-cortical circuits. The desynchronisation produced by ACh may be dependent upon reticular mechanisms initiated by cortical excitation. This hypothesis can also explain lack of action of ACh on the "cerveau isolé." In fact, Mollica(14) has demonstrated that it is impossible to provoke in this preparation the generalized desynchronisation with electrical stimulation of the cortex. Moreover, the synchronising effect, which is sometimes observed, can be caused by an action of the cortico-thalamic circuits whose activity is released from inhibitory control of midbrain reticular mechanisms.

Summary. 1) The effects on cerebral electrical activity (EEG) of acetylcholine, eserine, epinephrine, and amphetamine, injected by intracarotid route, are reported. Non-curarized unanesthetized rabbits and "cerveau isolé" preparation of the same animal were used. 2) In the unanesthetized non-curarized rabbit, acetylcholine, eserine, and amphetamine produced activation of the EEG; epinephrine, however, administered either by intravenous or intracarotid route, does not have the activating effect. 3) In the "cerveau isolé" preparation, acetylcholine does not provoke activation, while eserine and amphetamine are still effective. 4) These results are discussed in connection with the locus and the mechanism of action of the drugs.

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Accumulation of Organic Acids by HeLa Cells Infected with Type 4 Adenovirus.* (23113)

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That fluids from HeLa cell cultures infected with an adenovirus were more acidic than those from companion uninfected cultures was noted during early studies of the characteristics of these agents(1,2). Increased acidity of the media of infected cultures accompanied extensive cytopathogenic changes effected by the adenoviruses. Preliminary reports indicated that lowered pH of cultures infected with types 1-4 adenoviruses was a reflection of an increased quantity of lactic acid in the fluid, and the accumulation of acid was correlated with increased utilization of glucose by the infected cells(2).

This investigation was initiated to establish further the identity of acid or acids produced by uninfected and infected HeLa cells, and to determine whether an increased accumulation of acids other than lactic acid resulted from type 4 adenovirus infection. It is the purpose of this communication to report that lactic, pyruvic, acetic, and alpha ketoglutaric acids increase in fluids of HeLa cell cultures infected with this agent.

Methods. Tissue culture. HeLa cells were propagated in medium consisting of human serum, 40%, and Hanks' balanced salt solution (BSS), 60%, as described previously(3, 4). Cultures in tubes were initiated with 50,000 cells, and were used experimentally in approximately 24 hours. For infection, cultures were washed 3 times with 2 ml of Hanks' balanced salt solution per wash, and to each tube was added 0.8 ml of maintenance mixture composed of 67.5% Scherer's amino acid-vitamin mixture(5), 25% tryptose phosphate broth, and 7.5% chicken serum(4). To estimate pH of fluids, phenol red, 0.002%, was present in all media. *Viruses.* Type 4 aden-

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ovirus was propagated in HeLa cell cultures and its infectivity titer determined by methods previously described(3,4). To identify the acids and determine quantity of each present in the maintenance mixtures, cultures were infected with $10^{2.0}$ to $10^{3.5}$ 50% infectious doses (ID_{50}) of virus and the infected fluids or infected cells and fluids from cultures were pooled 4 to 6 days after infection. In each experiment fluids alone or cells and fluids (maintenance mixture) from uninfected cultures incubated for a similar period were also pooled. At the time that infected and uninfected cultures were harvested for determination of acids present, cell counts were not done since many cells in the infected cultures had fallen from the glass and an indeterminate number had lysed. Counts done on a large number of tube cultures after maintenance mixture had been added indicated that the variation in cell number between tubes prepared at the same time was less than 10%. *Initial identification and quantitative analyses of lactic acid* were done with culture fluids from uninfected and infected cultures using the method of Hullin and Noble(6). To identify further the carboxylic acids, chromatographic technics were employed using both cells and fluids from uninfected and infected HeLa cultures. To disrupt HeLa cells in suspension and obtain material for separation of acids, 1 ml of cell suspension was acidified to pH 2-3 with 10 N H_2SO_4 (usually a single drop of acid was required) and mixed with 2 g of celite by grinding with a pestle in evaporating dish. The mixture was then placed upon a 10 g celite column and the acids were eluted consecutively with a series of butanol-chloroform mixtures as described by Swim and Krampitz(7). Numerous experiments were done with prepared mixtures of known acids to determine the exact fraction in which each of the carboxylic acids would be eluted from the column under the conditions employed. Quantitative measurements for each acid fraction were made by titration with 0.01 N NaOH using phenol red as the indicator. During the titration procedure CO_2-free air was bubbled through the solution to obtain complete mixing. Lactic acid and keto-acids were also determined by colorimetric methods. Because the presence of phenol red in tissue culture fluids interfered with colorimetric determination of keto-acids, the data were valid only when samples for analysis were removed from the celite column. Contents of tubes considered to contain alpha keto-glutaric acid and possibly traces of succinic acid were pooled after separation from the celite column. The total volume was placed in an ice bath and an air jet was directed on the surface of the liquid until the chloroform layer had completely evaporated and the water phase was reduced to 1 or 2 ml. The sample was then diluted appropriately, the hydrazone prepared(8) and measured colorimetrically. By keeping the sample cool and retaining the compound as an acid and not the sodium salt, stability was maintained and reproducible results realized. When culture fluids were extracted with ether and the acids then separated on the column, results were in good agreement with values obtained when ether extraction was not employed. Therefore, extraction with ether was not used for most analyses. Compounds were further identified qualitatively by ascending paper chromatography using as solvent either a mixture of 85% secondary butyl alcohol, 5% formic acid and 10% water, or 75% methanol plus 25% saturated ammonium carbonate. With each chromatographic determination known acids were tested concomitantly with the homogenates from uninfected and infected cultures. These experiments were conducted at room temperature. Spots were developed by spraying the chromatograms with neutral brom cresol green indicator. *Glucose determination.* The quantity of glucose in maintenance mixture and in fluids from infected and uninfected HeLa cell cultures was measured by the method of Park and Johnson(9). Amount of glucose utilized by HeLa cells was computed by subtracting quantity of glucose present in the uninfected or infected culture fluid from that in maintenance mixture incubated at 36°C for the same period as the cultures.

Results. Comparison of fluids from uninfected and adenovirus infected HeLa cell cultures clearly indicates the greater acidity of maintenance fluid from the infected cultures.

TABLE I. Quantity of Lactic Acid Produced and Glucose Utilized by Uninfected and Type 4 Adenovirus Infected HeLa Cells.

Exp. No.	HeLa cell culture*	Lactic acid produced, μM	Glucose utilized, μM
1	Uninfected	1.4	
	Infected†	4.7	
2	Uninfected	4.7	
	Infected†	7.0	
3	Uninfected	.9	1.4
	Infected†	3.2	6.4
	Infected‡	2.5	4.8

* Incubated at 36°C for 6 days.
† Infected with $10^{3.0}$ ID_{50}.
‡ " " $10^{2.0}$ " .

The progressive decrease in pH of infected cultures to a pH of 6.5-6.8 has been correlated with viral multiplication and subsequent cytopathogenic changes.‡ Chemical analysis of fluids from control and infected cultures indicated that a marked accumulation of lactic acid occurred. Results from studies carried out with the type 4 adenovirus are presented in the tables. Data from 3 typical experiments presented in Table I illustrate the range of differences between quantity of lactic acid in fluids from infected and uninfected cultures. Studies were next done to determine whether glucose was utilized by viral infected cells in amounts commensurate with the quantity of lactic acid which accumulated; the results from one such study presented in Table I indicate that this was the case. It should be noted, however, that in control and infected cultures the amount of glucose metabolized was 3 to 4 times more than could be accounted for by the quantity of lactic acid produced. The results of these limited studies suggest that with HeLa cells, uninfected or infected, although lactic acid is a principal end-product of glucose metabolism mechanisms other than anaerobic glycolysis are also involved.

The data presented in Table I were the results of determinations done on materials obtained by pooling 2 to 4 cultures. Analyses were next done with pools of cells and fluids made from 20 to 30 uninfected or infected cultures. For viral infection $10^{3.5}$ ID_{50} per culture of type 4 adenovirus was employed. Cultures were harvested 4 days after viral infection when practically all cells of the infected cultures had undergone characteristic cytopathic changes. To determine whether a variety of carboxylic acids accumulated as a result of viral infection, acids were separated and measured as described above. Glucose determinations were not done in these experiments. The results from 5 individual experiments are summarized in Table II. These data further point out the increased amount of lactic acid found in viral infected cultures. In addition, pyruvic and alpha ketoglutaric

TABLE II. Quantity of Organic Acids in Uninfected and Type 4 Adenovirus Infected HeLa Cell Cultures Determined by Chromatographic and Colorimetric Technics.

		Micromoles acid* determined by						
		Titration technic†				Colorimetric technic		
Exp. No.	HeLa cell culture	Acetic	Pyruvic	Lactic	α-keto-glutaric	Pyruvic	Lactic	α-keto-glutaric
1§	Uninfected	6.2	2.7	5.4	1.8	nt‡	nt	nt
	Infected**	9.6	3.1	7.4	2.2	nt	nt	nt
2‖	Uninfected	5.0	0	3.9	.7	nt	nt	nt
	Infected**	5.9	4.5	10.1	1.5	nt	nt	nt
3¶	Uninfected	6.2	1.0	3.1	.1	nt	3.3	nt
	Infected**	10.5	3.7	6.2	1.4	nt	6.1	nt
4¶	Uninfected	5.2	.2	6.1	.6	.9	6.4	.8
	Infected**	9.9	1.2	13.2	2.1	1.4	nt	2.3
5¶	Uninfected	1.5	0	2.1	0	0	2.9	0
	Infected**	4.6	2.6	5.4	1.5	2.2	5.9	1.6

* Acids separated on a celite column. † Titrated with .01 N NaOH. ‡ nt = not tested. § Cultures incubated at 36°C for 3 days. ‖ Cultures incubated at 36°C for 4 days. ¶ Cultures incubated at 36°C for 6 days. ** Infected with $10^{2.5}$-$10^{3.0}$ ID_{50} of Type 4 adenovirus.

‡ Unpublished data.

TABLE III. Identification by Paper Chromatography of Organic Acids in Uninfected and Type 4 Adenovirus Infected HeLa Cell Cultures. Comparison of R_f values and known acids.

Solvent	Acid determined	R_f values: Known (control)	R_f values: HeLa culture* Uninfected	R_f values: HeLa culture* Infected
Butyl alcohol - formic acid - water†	Acetic	.08	.08	.08
	Lactic	.8	—	.8
	α-ketoglutaric	.05	.05	.05
Methanol - saturated $(NH_4)_2CO_3$‡	Acetic	.19	—	.19
	Pyruvic	.58	—	.58
	Lactic	.72	—	.71

* Materials from Exp. 5, Table II.
† 85% secondary butyl alcohol, 5% formic acid and 10% water.
‡ 75% methanol and 25% saturated $(NH_4)_2CO_3$.

acids were consistently present in greater quantity in cultures infected with type 4 adenovirus; acetic acid also accumulated in greater amounts in the majority of infected cultures although the increase over that measured in uninfected cultures was not as marked as that of the other acids identified. The increased accumulation of lactic, pyruvic, and alpha ketoglutaric acids was also demonstrated by colorimetric methods.

The identity of these carboxylic acids was further confirmed by the use of paper chromatographic technics. An identification of compounds in culture homogenates from one experiment (Table II, Exp. 5) is summarized in Table III. The R_f values of the acids from uninfected and viral infected cultures corresponded very closely with R_f values of known solutions of lactic, pyruvic, acetic, and alpha ketoglutaric acids. On paper, a spot which corresponded to succinic acid could also be identified. This acid could not be measured in the eluate from the celite column because the small amount present was probably in the fraction with alpha ketoglutaric acid. There were no spots on the chromatograms which could not be identified. These data indicate that the acids obtained by fractionation of culture materials on the celite column were indeed the acids considered to be present and lend strong support to the findings described above.

Discussion. That adenovirus infection of HeLa cells results in an accumulation of organic acids in the culture media is demonstrated by the experimental data exemplified in this paper with type 4 viral infection. The acids identified as being present in uninfected and infected culture fluids were lactic, pyruvic, acetic, alpha ketoglutaric, and probably very small amounts of succinic. The increased accumulation of lactic acid and concomitant increased utilization of glucose by infected cells imply that adenovirus infection of HeLa cells results in a stimulation of glycolysis. The pathways of carbohydrate metabolism by HeLa cells have not yet been elucidated, so that the mechanism by which pyruvic, acetic, and alpha ketoglutaric acids accumulate and their relationship to the mode of viral action is not clear. The presence of the enzymes of the Krebs citric acid cycle in normal HeLa cells has recently been demonstrated(10), but their role in uninfected or adenovirus infected HeLa cells has not been studied.

The evidence presented does not permit the conclusion that the increased glycolysis and accumulation of carboxylic acids is an inherent part of viral synthesis. The phenomenon described by this investigation may rather reflect cell injury resulting from viral infection and could perhaps be initiated also by cell damage due to non-viral noxious agents. It is clear, however, that injury of cultured cells in general and HeLa cells in particular by a variety of other viruses, such as western equine encephalomyelitis(11), poliomyelitis (12-14), Coxsackie§, ECHO§ and Newcastle disease viruses,¶ does not cause increased accumulation of acids. Conversely, infection

¶ Unpublished data.

§ Steigman, A. J., Benyesh, M., Brown, R., and Melnick, J. L., personal communication.

with these agents results in a decreased production of acid. It is noteworthy that infection of HeLa cells by the latter viruses listed culminates in death and lysis of the host cells. In contrast, adenoviruses do not appear to lyse infected HeLa cells(2) and it has been suggested that these respiratory tract viruses do not actually kill the cells in which they multiply(2). It can be deduced, therefore, that the increased glycolysis and accumulation of carboxylic acids require a particular type of viral infection or cell injury. Whatever may be the mechanism of the increased accumulation of organic acids and utilization of glucose, the evidence is clear that the initiation of this phenomenon is an intrinsic characteristic of the adenoviruses and a constant effect upon HeLa cells which they infect.

Summary. Fluids from cultures infected with type 4 adenovirus were markedly more acid than fluids from non-infected control cultures. The lowered pH of infected cultures resulted from an accumulation of lactic, pyruvic, acetic and alpha ketoglutaric acids. The acids were separated by chromatography on a celite column, and measured by colorimetric and acid-base titration technics. Confirmation of the identity of the organic acids was obtained by paper chromatography. Concomitant with the increased accumulation of acid in fluids of infected cultures, an increased utilization of glucose by cells of the virus-infected cultures occurred.

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Guanine and Guanosine Deaminase Activity of Rat Mammary Gland Homogenates Through Pregnancy and Lactation.* (23114)

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Kirkham and Turner(1,2) reported changing levels of rat mammary gland DNA and PNA during pregnancy and lactation and in the mammary gland stimulated to growth with estrogen and progesterone. In rabbit mammary gland Yamamoto and Turner(3) showed increases in total DNA as glandular growth occurred. McShan *et al.*(4) have shown very striking increases in PNA content of pigeon crop gland following treatment with lactogenic hormone. Williams and Turner (5) reported apparent involvement of PNA in the action of lactogenic hormone on the mammary gland. From these studies PNA

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is implicated in the process of milk synthesis and increases in mammary gland PNA which have been noted probably correspond roughly to the amount of synthesis occurring in the mammary gland. There have been relatively few studies concerning the deaminases of guanine and guanosine. Schmidt(6) and Wakabayasi(7) reported separation of the two enzymes. Plentl and Schoenheimer(8) demonstrated guanine deaminase activity in various rat tissues. Block and Johnson(9) found guanine deaminase in rat skin. Schmidt (6) isolated guanine deaminase from rabbit liver. Even less work has been reported concerning guanosine deaminase; in fact, Kalckar doubts the existence of such an enzyme, believing the deamination of guanosine to be carried out by guanine deaminase and nucleoside phosphorylase. Guanosine deamination has been demonstrated in rabbit liver preparation by Schmidt(6) and in various tissues of the rabbit, cat and pig by Wakabayasi(7).

Methods and materials. Female albino rats, weighing approximately 150 g, were used. The mammary glands were studied in 4 physiological states; virgin non-growing, growing during pregnancy, lactating and involuting following lactation. The experimental animal was sacrificed, the mammary tissue rapidly dissected free and chilled on ice. The mammary tissue was then homogenized for 3 minutes in a Waring blendor in the cold with 9 volumes of isotonic NaCl, strained through 4 layers of cheese cloth and assayed immediately for guanine and guanosine deaminase activity. The assay was carried out using the manometric technic described by Zittle(10) for determination of liberated ammonia. The reaction flask contents for all determinations were as follows: 1.4 ml of 1.15 M $NaHCO_3$ gassed with CO_2, 1 ml of mammary gland of homogenate, and 5 mg of substrate. The gas phase was 100% CO_2 and a bath temperature of 38°C. The final flask volume was 3.4 ml and the flask pH 7. The flask contents were assayed for DNA content by the method of Webb and Levy(11). A DNA standard was used which assayed 7.8% phosphorus.

Results. Rat mammary gland homogenates were found to deaminate both guanine and guanosine in all physiological states studied. The rate of guanine deamination was consistently some 3 times that for guanosine.

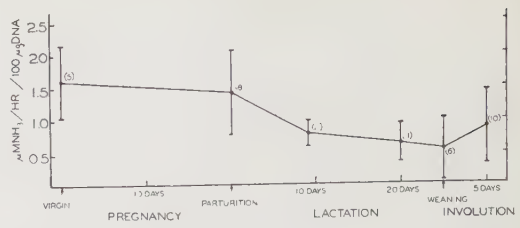

FIG. 1. Guanine deaminase activity during pregnancy, lactation and involution in rat mammary gland.

A change in rate of deamination was noted when mammary tissue from pregnant animals was compared to that from animals in lactation (Fig. 1). Guanine deamination declined from a mean value of 1.42 μM/hr/100 μg DNA during pregnancy to 0.72 μM/hr/100 μg DNA during lactation. This change was highly significant ($P < .0025$).

This same change in rate of deamination was noted for guanosine deamination when pregnancy and lactation were compared. Guanosine deamination declined from a mean of 0.44 μM/hr/100 μg DNA during pregnancy to a value of 0.24 μM/hr/100 μg DNA during lactation. This change was also highly significant ($P < .0025$).

Summary. Guanine deaminase activity and guanosine deaminase activity of rat mammary homogenates were studied through pregnancy, lactation and involution. Guanine and guanosine deamination occurred in mammary gland homogenates during all physiological states studied. The rate of deamination of guanine and guanosine exhibited a marked drop from the level of pregnancy to a lower level throughout lactation.

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Pipetting Machine with Rapid Preset. (23115)

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Several immunochemical procedures require the addition of less than cc volumes of reagents to large series of tubes. In studies on production of hemolysin by mice or other laboratory animals following the method described by Taliaferro(1) large series of serum dilutions must be made. Pipetting machines are available* for repetitive delivery of fluid volumes but resetting for some new volume requires careful recalibration and adjustment. Ordinarily, there is not enough time for these manipulations during the course of a given test. The machine described here was constructed to reduce the time required for adding quantities such as 0.02, 0.04, 0.06 cc, etc. to sets of 20 to 30 tubes in the serial dilutions of serums for estimation of their hemolysin content.

Methods. Fig. 1 is a general view of the assembly and shows presetting button (white), toggle switch (black) for operating the machine and other features described in detail in Fig. 2. Details of construction of the plunger and associated parts of the machine are illustrated in Fig. 2, I. The stainless steel plunger, A, Fig. 2, I, was made 0.874 cm in diameter and displaces a 3 cc volume when inserted a distance of 5 cm in the fluid reservoir, B. Fluid volume displaced is directly proportional to movement of the plunger. Control of the forward movement and hence of the fluid displaced is provided in the preset counter by means of the micro-switch, C. Counting rate is determined by the 4-sided cam, D, and with the gear ratio and plunger shown here the preset counter records one count for each 0.01 cc delivered. Tapped holes in various positions around the cam allow one to obtain a larger or smaller number of counts per unit quantity of fluid delivered. Worm gears, E, with a 60 to 1 ratio and the pinion gear, F, advances the rack at the rate of 4.0 cm per revolution. By revolving the slotted ram, G, in a counterclockwise direction the rack and pinion become disengaged allowing the plunger assembly to be withdrawn for reloading.

An exploded view of the preset counter indicating the relationships between its 4 principal parts is shown from left to right in Fig. 2, II. Number of counts and thus volume to be delivered is determined by the setting of the dial, 1, which positions the stop block, 2. Immediately to the right of the stop in Fig. 2, II, is shown a single unit combining the following 4 structures: the cam, 3, that actuates the microswitch controlling the motor-drive; the positioning stop, 3S; the indexing tongue, 3L; and the axle of the instrument. The controlling element, shown at far right in Fig. 2, II, during normal operation lies in the position indicated by x - - - x. This structure is shown in place in Fig. 2, III. The ratchet wheel 4R and the toothed wheel 4S are machined as a single unit and are actuated by the relay armature, 5. The wheel, 4S, has 70-1/32 inch slots spaced about its periphery and the ratchet, 4R, has 70 teeth which correspond with the divisions

* Brewer automatic pipetting machine, Baltimore Biol. Lab., Baltimore, Md., and Sterling Automatic Pipette, Ivan Sorvall, N. Y. are 2 of the available mechanical pipettors in use in our laboratory.

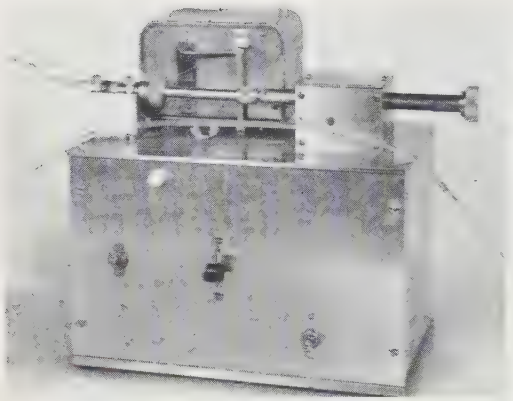

FIG. 1. Front view of pipetting machine showing preset button (white) and operating toggle switch.

on the dial, 1.

When the current which operates the relay is interrupted by the microswitch, C, the indexing tongue, 3L, engages the wheel 4S to rotate the cam. A thrust spring holds the parts of the controlling assembly together and a spiral spring, 6, loads the cam shaft to return it to the stop. This action is triggered by the operator as the reset lever, 7, is depressed thus disengaging the tongue, 3L. Resetting for repeated delivery of a given volume is done by pressing the reset lever while selecting a new volume for repetitive delivery is done simply by turning the dial to a new position.

Time required to deliver less than cc volumes of fluids to large series of tubes has been reduced and fatigue associated with frequent pipette refillings has been essentially eliminated by the use of the pipetting machine just described. Since the volume delivered is directly proportional to the linear movement of the plunger, the accuracy of the instrument is related to the accuracy of machining the plunger. Aside from its reliability the great advantage of the machine just described lies in the rapidity with which a new volume setting can be made.

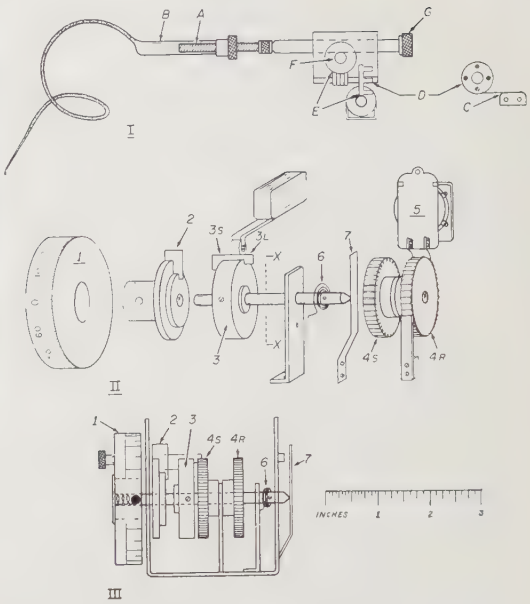

FIG. 2. Details of construction of pipetting machine; part I is shown half scale, parts II and III are shown full scale.

Summary. A pipetting machine designed for the repetitive delivery of fluid volumes in the range of 0.01 cc to 0.60 cc has been described. A counter and its associated parts drives an accurately made stainless steel plunger forward displacing the fluid in a glass cylinder at such a rate that one count represents a delivered volume of 0.01 cc. Any given number of counts up to 60 may be selected allowing the operator to deliver that volume repeatedly to a large series of tubes. Accuracy of delivery is ensured since the linear movement of the plunger is directly proportional to the volume delivered; time is saved in pipetting operations involving large series of tubes and the fatigue of frequent pipette refillings is reduced.

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Production of Peptone Shock in Mice Following Administration of *H. pertussis* Vaccine. (23116)

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Previous studies(1,2,3) in this laboratory have shown that brucella endotoxin is lethal for mice within 24 hours after injection. Mice were protected against endotoxin with cortisone and chlorpromazine. It was also demonstrated that the sensitivity of mice to endotoxin could be enhanced by pertussis vaccine. Further investigations(4,5) in larger animals revealed that endotoxin caused shock with the pooling of venous blood. This shock simulated that following the injection of peptone, histamine, trypsin and other agents(6).

The purpose of the present investigation was to compare the action of peptone with endotoxin when injected into mice. Of particular interest was a study of the enhancing effect of pertussis vaccine on peptone, as well as the protection against peptone offered by cortisone, chlorpromazine and an antihistaminic agent.

Materials and methods. Groups of ABC male and female mice, and Swiss-Webster female mice, weighing 20-30 g were employed.* A 30% suspension by volume in distilled water of proteose-peptone (Difco) was used. The suspension was autoclaved after constitution and stored at 4°C. A standardized *Hemophilus pertussis* vaccine (Lederle) was administered to the mice. The antihistaminic was diphenhydramine hydrochloride (Benadryl), and fresh saline suspensions of cortisone (Merck) and chlorpromazine were injected. Unless otherwise stated, all materials were administered intraperitoneally.

Results. 1. *Enhancing effect of H. pertussis vaccine on lethality of peptone.* All populations of mice used in these studies were resistant to doses of peptone up to 150 mg. However, when mice were given 0.2 ml of pertussis vaccine 6 days prior to receiving peptone, the peptone was lethal. The results of a typical experiment are as follows: To each of 20 Swiss-Webster female mice 150 mg of peptone was administered without any demonstrable ill effects. However, when 0.2 ml of pertussis vaccine was given to each of 20 mice 6 days prior to the peptone, 18 out of the 20 mice died within 24 hours.

Increased susceptibility of the mice to peptone could be detected as early as one day after the administration of pertussis vaccine, increasing to a maximum sensitivity within 5-6 days, and then disappearing in 21-28 days. While death within 24 hours was used as the criterion for susceptibility to peptone, most of the mice died within a few hours after the injection of peptone, many within 30 minutes. Just before death the mice exhibited respiratory difficulty like that usually observed after the injection of histamine. The animals quickly became inactive with ruffling of the fur. Respiratory difficulty was manifested by the animals rubbing their noses with their paws; slowing and deepening of the respirations; inspiratory difficulty; and a progressive respiratory failure. At necropsy, serous fluid was present in the peritoneal cavity. The lungs were distended, but collapsed when the thorax was opened. No gross hemorrhagic areas were noted in the lungs. The heart continued to beat after respiration had ceased.

The foregoing phenomenon of increased susceptibility was also demonstrated in ABC female and male mice, and in Ace F_1 female mice.

2. *Protection by an antihistaminic agent against increased susceptibility to peptone.* As seen in Table I, 4 groups of Swiss-Webster female mice were used. A control group was given 150 mg of peptone alone; a second group was pretreated with 0.2 ml of pertussis vaccine, and then given 150 mg of peptone 6 days later; a third group was treated similarly as the second group, except that 3½ hours and 10 minutes before, as well as 3

* The ABC mice were obtained from Dr. John Bittner, Division of Cancer Biology, University of Minn.

TABLE I. Protective Effect of Benadryl in Swiss-Webster Mice Sensitized to Peptone by Prior Treatment with Pertussis Vaccine.

No. of mice	Treatment	Outcome*
20	Peptone alone	0/20
20	Pertussis vaccine + peptone	18/20
20	*Idem* + Benadryl	0/20
10	Peptone + Benadryl	0/10

* Numerator denotes No. of deaths. Denominator denotes total No. of animals.

hours after the peptone injection, 50 μg of Benadryl was administered; a fourth group was given the combination of peptone and Benadryl without prior treatment with pertussis vaccine.

It will be noted that no deaths occurred in the mice treated with Benadryl. It was of interest that in the Benadryl-protected mice, the animals appeared inactive and the fur was ruffled shortly after the injection of peptone, but they soon recovered completely.

3. *Protection by chlorpromazine against increased susceptibility to peptone.* An experiment similar to that described with Benadryl was carried out, except that an attempt was made to protect sensitized Swiss-Webster female mice with chlorpromazine. When 10 sensitized mice were given 50 μg of chlorpromazine 2 hours before, 15 minutes before, and 30 minutes after the injection of 150 mg peptone, none of the mice died. Nine of 16 control mice died within 24 hours. Chlorpromazine appeared to offer the same degree of protection as Benadryl.

4. *Protection by cortisone against increased susceptibility to peptone.* A similar group of experiments was designed to test the protective action of cortisone. When 10 mice were sensitized with pertussis vaccine as described, and then given 150 mg of peptone, death was prevented by treating the animals with one intramuscular injection of 0.5 mg cortisone 6 hours prior to the peptone. Seven of 10 mice expired within 24 hours after the injection of peptone. Under these circumstances, cortisone protected the mice similar to Benadryl and chlorpromazine.

Discussion. The mechanism which makes mice more susceptible to peptone after treatment with pertussis vaccine is not understood. Pertussis vaccine increases susceptibility of mice to histamine(7). In view of this it would be tempting to conclude that histamine is released by peptone in these more susceptible mice. Peptone does liberate histamine in the dog(8). In addition, the peptone death in mice simulates the respiratory failure induced by histamine, and protection is offered to the mice by those agents which are known to block histamine activity. However, it cannot be assumed on the basis of available evidence that these drugs protect the peptone-susceptible mice by a direct antihistaminic effect. Histamine is probably not the only factor involved, since it has been demonstrated in this laboratory that Swiss-Webster female mice are made more susceptible to histamine by pertussis vaccine, whereas ABC female mice remain resistant. On the other hand, increased sensitivity of both populations of mice to peptone follows administration of pertussis vaccine. The suggestion that increased sensitivity of mice to histamine due to pertussis vaccine results from altered adrenal activity remains to be proved(9).

Summary. Populations of ABC and Swiss-Webster male and female mice were found to be resistant to peptone, but an increased susceptibility to peptone with a lethal outcome could be produced by prior treatment with pertussis vaccine. Mice rendered susceptible to peptone with pertussis vaccine could be protected with the antihistaminic agent, Benadryl, with chlorpromazine and with cortisone. The possible role of histamine in the increased susceptibility of mice to peptone, produced by pertussis vaccine, has been discussed.

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Cultivation of Spirochaetes from Spinal Fluids of Multiple Sclerosis Cases and Negative Controls.* (23117)

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Since 1838 when multiple sclerosis was first recognized as a demyelinating disease, many attempts have been made to establish its etiology. There were many conflicting theories. Originally it was thought that multiple sclerosis was due to toxins that destroy the myelin sheaths; second, that it was an allergic or biochemical reaction. Buzzard(1) advanced the theory that due to resemblance of multiple sclerosis to cerebrospinal syphilis, the causative agent may be an organism of the Treponema family. He did not succeed in demonstrating the suspected organism. Steiner and Kuhn(2) found spirochaetes in brain tissues and spinal cords of patients who had died of multiple sclerosis. Adams, Blacklock and M'Cluskie(3) were able to demonstrate spirochaetes under darkfield illumination while examining fluid from lateral ventricles of monkeys and rabbits injected with material from multiple sclerosis cases. This fluid was examined several months after inoculation when the animals developed signs of illness. The spirochaetes were identical in appearance with those described by Steiner. All attempts to cultivate the spirochaetes failed. Collins and Noguchi(4) tried to grow the organism by inoculating spinal fluids from multiple sclerosis cases into Noguchi culture medium. They were unable to get positive results. In 1951, I devised a culture medium in which spinal fluids from known cases of multiple sclerosis were inoculated, and, after one to 2 weeks incubation, few spirochaetes were found under phase and darkfield illumination. Cultures from spinal fluids of healthy individuals and of patients with other neurological diseases were negative and remained sterile after one year's incubation. The original culture medium did not show many organisms per field; growth was poor. To obtain a better growth, the culture medium was modified, and subcultures from the original positive culture and fresh spinal fluid reinoculated into the modified medium showed heavy growth of spirochaetes after several days' incubation.

Methods. Stoppered test tubes are sterilized by autoclaving, then sterilized again in hot air sterilizer for one hour at 180°C. About 10 ml of spinal fluid are collected into

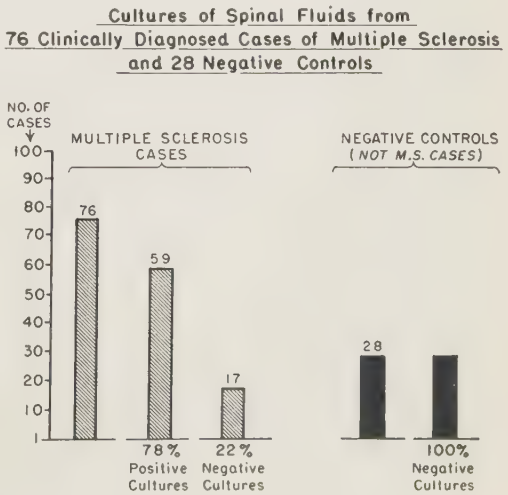

FIG. 1.

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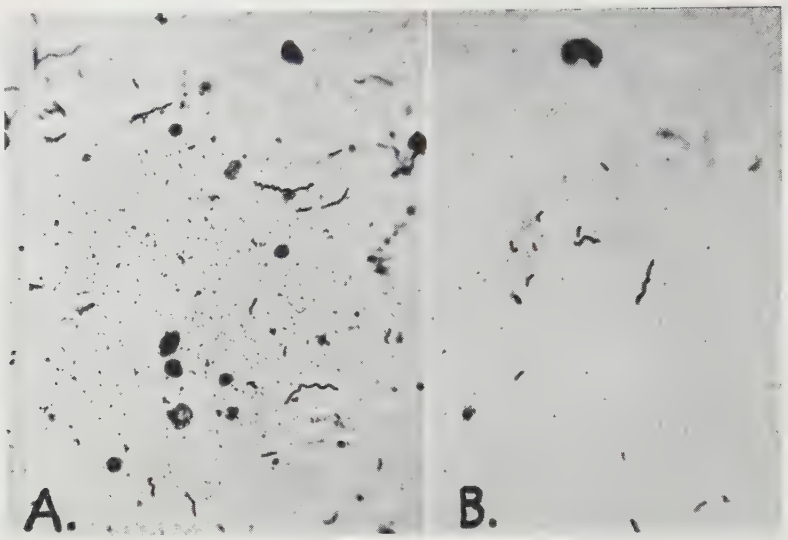

FIG. 2. Photomicrographs of 2 fields, A and B, from a smear of culture of spinal fluid from M.S. case A.M. after staining by the silver nitrate-tannin method. Magnification × 970.

cooled sterile tubes and centrifuged at 1500 rpm for 1½ to 2 hours. If red blood cells are present or the fluid is xanthochromic, it is not suitable for cultivation. The supernatant fluid is discarded, leaving a few drops mixed with sediment and, by a Wright pipette, inoculated into the following modified culture medium: Distilled water 1000 ml, Brewers Thioglycollate Medium (Difco #B236) 20 g, Bacto-Asparagin (Difco) 0.4 g, L-Cystine (Difco) 0.2 g, Bacto-Peptone (Difco) 1 g. Boil 20 minutes, cool to 37°C, add—rabbit sera 20 ml, Wassermann negative human sera 60 ml. Filter through Selas 03 filter, pour into sterile test tubes and inoculate with spinal fluid as described above. To secure anaerobiasis, about 3-5 ml of sterile paraffin oil (50%) and yellow vaseline (50%), previously sterilized one hour in autoclave and cooled, are poured into the inoculated tubes. Inoculated cultures are incubated at 30°C and examined once a week under phase microscope. Silver nitrate-tannin method is used for staining smears from positive cultures.

Results. The results obtained by culturing the spinal fluids from 76 cases of clinically diagnosed multiple sclerosis and 28 individuals not recognized as having multiple sclerosis are summarized in Fig. 1.

Morphology. By phase- and darkfield-microscopy, micro-organisms are observed which vary from 10-22 μ in length, about 1 μ thick. They have wide spirals; some have a loop at one end while others resemble a tennis racquet. Photomicrographs (2a and 2b) of stained organisms are reproduced in Fig. 2. They are actively motile. They also have a tendency to dive into the fluid. The spirochaetes are identical in appearance to those found in brain tissue by Steiner, who named them Spirochaeta Myelophthora.

Summary. New culture medium was devised in which it was possible to grow spirochaetes from spinal fluids of cases of multiple sclerosis. Photomicrographs of the cultured spirochaetes are reproduced. The results of cultivation are given in Fig. 1.

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Transformation of Fibroma into Myxoma Virus in Tissue Culture. (23118)

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Transformation of Shope fibroma virus into that of myxoma was first reported by Berry and Dedrick(1,2) who used heat-inactivated myxoma virus as the transforming agent. Their findings were confirmed by other investigators(3,4). As summarized by Smith(4), results of experiments performed in rabbits were "troublingly irregular" and usually not repeatable at will. Experiments presented below demonstrate that the Berry-Dedrick phenomenon can take place in cultures of rabbit tissues, both testis and kidney. They also give some indication that positive results might be obtained with greater regularity *in vitro* than *in vivo*.

Materials and methods. Tissue cultures. General methods of growing explants of rabbit testis presented elsewhere(5) have been modified in all but 2 of the present experiments involving this tissue by the use of medium 199(6) containing 20% of inactivated horse serum. In remaining experiments, tissue cultures consisted of trypsinized(7) rabbit kidney cells. Nutrient medium for the latter cultures consisted of 0.5% lactalbumin hydrolysate, as described by Melnick(8). All cultures were in roller tubes, and all media contained penicillin and streptomycin. *Rabbits and precautions in handling.* Dutch rabbits were used as the source of kidneys and testes for cultures except for a single experiment performed with testis tissue from a cottontail rabbit. Immature New Zealand Whites were used for testing infected tissue culture fluids. Test rabbits were kept in 2 rooms. The one contained animals for the first 4 days after inoculation; the other, rabbits transferred from the first after this time, but before they were likely to develop systemic infection with myxoma virus. The rabbits were free from hemophagus arachnids. Insect sprays were used in both rooms. Most rabbits which developed myxoma were killed within 24 hours of the time it first became apparent. *Virus.* The Patuxent strain(9) of fibroma was employed throughout. Methods of virus titration in skin of domestic rabbits have been described(10). *Preparation of transforming agent.* TAM or Transforming Agent Myxoma was prepared from skin tumors of domestic rabbits which had developed myxomatosis 6 or 7 days after intracutaneous inoculation of myxoma virus. Tumors were cut away from the outer skin, ground with sand, made into 10% suspensions with Hanks' balanced salt solution containing 10% horse serum, then centrifuged several times at 3,000 rpm. Supernates from these preliminary centrifugations were centrifuged at 40,000 rpm for 40 minutes. Sediments from the higher speed centrifugation were suspended at 1/10 the previous volume, dispersed by a micro-blender, sealed in glass tubes, and submerged in water bath at 66-67°C for 40 minutes. Two lots of TAM were prepared differently, but with equally successful results as shown in Table I. In TAM 8 the myxomas were made in 20% suspension originally and were not submitted to high speed centrifugation. TAM 10 was prepared as first described, except that inactivation was limited to 12 minutes at 65°C. *Performance of experiments.* Live fibroma virus, contained in a 1:10 suspension of cottontail or rabbit fibroma in Hanks' solution or in undiluted tissue culture fluid from previous passage of the agent (Table I), was mixed with TAM in proportions of 0.1 ml of TAM (0.5 ml for TAM 8) to 0.2 ml of fibroma virus suspension for each roller tube (16 x 150) inoculated. Mixtures of TAM and virus were kept 15 minutes at room temperature before inoculation. Tubes receiving the mixture contained a final volume of 2.1 ml, and were incubated in a roller drum at 36°C. Nutrient fluids were harvested and pooled twice, or preferably 3 times a week. Fresh fluids, containing TAM in amounts originally inoculated, were then added. Test rabbits were inoculated intracutaneously with 0.5 ml from

TABLE I. Details of Positive Experiments in Which Fibroma-Myxoma Transformations Occurred in 11 Experiments.

Exp.	Rabbit tissue culture	Source fib. vir.	TAM† lot No.	Time of transformation,‡ days after T.C. inoc.
BD 1*	Testis	5P T.C. Cot. testis	T 1	13*
2	"	3P T.C. Rab. testis	2	7
3	"	Rab. fib.	3	10
6	"	Cot. fib.	5	6
7	"	*Idem*	4	6
32	"	"	8	9
39	"	"	8	5
37	Kidney	1P T.C. Rab. testis	10	2
38	"	1P T.C. Rab. kidney	10	11
41	"	1P T.C. Rab. testis	10	9
43	"	*Idem*	10	5

* Only exp. in which testis culture used was from cottontail and not domestic rabbit tissue.

† Transforming agent myxoma.

‡ Refers to time that myxoma virus was first detectable by testing supernatant fluids from tissue cultures in the skin of a rabbit.

each pool of infected fluid. The same rabbit was usually inoculated with fluid from a number of experiments. If the animal developed only local lesions after 12 days of observation, all fluids were considered negative, *i.e.*, no transformation of fibroma to myxoma had occurred. However, if the test rabbit developed a systemic infection in 6 or 7, but occasionally to 9 days after inoculation, samples of the original supernatant fluids, stored in dry ice, were again tested, each in a single rabbit. Generalized myxomatosis was first indicated by appearance of white pus at corners of the eyes, followed by swelling of eyelids, tracheal rattles, and enlargement of regional lymph nodes within 24 hours. At this time most rabbits were killed and a specimen taken of the swollen, jelly-like conjunctival tissues. These specimens were fixed in 10% formalin. A few rabbits, not killed, succumbed within 3 or 4 days of the first appearance of pus in the eyes.

Results. TAM. Each lot was tested for possible presence of infective virus particles by injection of 1 ml into several sites in the skin of rabbits. Some rabbits were likewise inoculated intraperitoneally. All rabbits remained healthy for the 2-week period of observation, without development of local lesions. Lots 4 through 10 were carried in tissue cultures, in the same manner as in actual transformation experiments, only without the presence of live fibroma. Fluids harvested from these passages produced neither local nor systemic reactions in rabbits inoculated with them, indicating that inactivated particles had not been restored to activity. TAM represented myxoma virus heated 40 minutes, but it was found that no infective virus was recoverable after 3 minutes exposure to the temperature used. The effectiveness of 65°C and above for inactivating myxoma virus has been attested by others (2,3,4). Seven of 10 lots of TAM prepared were effective in at least one successful experiment (Table I).

Transformations in tissue culture. Table I demonstrates that transformation of fibroma virus, as indicated by appearance of infectious myxoma virus in supernatant fluids, took place in 2 to 14 days of inoculation of the tissue cultures. In the group of 4 experiments performed in cultures of rabbit kidney, fibroma virus inoculated at the start had already grown in the presence of TAM for 5 to 6 days, since fluids used as a source of the agent were from previous experiments in which no transformation had occurred. Experiments usually continued for about 2 weeks. At the end of this time cultures might show degenerative changes whether they had been inoculated or not. Once fluids became positive for myxoma virus they remained so until termination of the experiment, with one exception. In experiment BD 32 the testis cultures used were usually good, so that the experiment was carried on for 32 days, at which time the cultures were 38 days old. As shown in Table II, fluids harvested 9, 11, 14, and 16 days after inoculation were positive for myxoma virus and had virus titers of from 10^{-4} to 10^{-5}. Fluids harvested before and after the 9-16 day period had lower titers and contained fibroma virus only.

TABLE II. Results of Tests and Titrations on Supernatant Fluids Taken Serially from Tissue Cultures of Two Transformation Experiments.

Exp.	TAM added	Results of tests and titrations in rabbit skin: Days after inoculation of tissue culture — 4	7	9	11	14	16	18	25	32
BD 32	With fibroma virus	FIB* 10^{-2}†	FIB 10^{-3}	MYX 10^{-4}	MYX 10^{-4}	MYX 10^{-5}	MYX 10^{-5}	FIB 10^{-2}	FIB 10^{-1}	FIB 10^{-1}
35	4 days after fibroma virus	FIB 10^{-1}	FIB 10^{-2}	FIB 10^{-3}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-1}	FIB 10^{-1}

* Type of disease produced in test rabbit. † Virus infectivity titers of the fluids tested.

Results of another experiment, BD 35, are also given in Table II. This experiment was set up in parallel with BD 32, using the same tissue cultures and virus preparation, the only difference being that TAM was not added until 4 days after the inoculation of fibroma virus. The tissue cultures grew equally well in both experiments. Myxoma virus was never recovered from fluids of the BD 35 experiment and virus titers never rose above 10^{-3}. It thus appeared that when transformation did take place, virus titers became elevated. One might explain that fibroma and myxoma give similar type tumors on titration in the skin of a rabbit.

Discussion. Present experiments indicate that transformation of fibroma into myxoma virus may be accomplished with some variation in the type of tissue culture, preparation of transforming agent, and in the source of fibroma used in an individual experiment. Two conditions appeared to be essential. One was addition of the transforming agent (TAM) at the same time as the live virus and secondly, that the tissue cultures have a good growth of cells to support a vigorous proliferation of virus. Negative experiments have not been described; a feature common to the majority, however, was a poor growth of supporting cells.

Experiments have been presented as if transformations took place entirely in tissue culture. They probably do. However, one must consider that final stages of the process could take place in the test rabbit, since the rabbit inoculum contains both live fibroma virus and residual TAM. Several facts suggest that transformations originated in the course of virus proliferation within the roller tubes. For example, once supernatant fluids became positive in an experiment, successive fluids were generally positive, and repeated tests on each fluid were also positive. Test rabbits given tissue culture fluids before this time did not develop myxoma, although such fluid contained TAM and fibroma virus. Transformations in rabbits alone have usually not been as consistent as judged by the experience of other investigators(4). Supernatant fluids from successful experiments presumably contained a mixture of fibroma and myxoma viruses. A small amount of the latter agent may be sufficient to outgrow an excess of the former, especially in a rabbit. Test animals usually developed myxoma within 6 days of inoculation. This represents nearly a minimum incubation period for the strains of virus and of rabbit employed. The shortness of the time suggests that the infective myxoma particles were in the tissue culture fluids inoculated and that the rabbits had not acquired accidental infections. So far there has been no evidence of accidental infections and control rabbits, in the same animal rooms, have remained free of myxoma. It is likely that myxoma is transmissible directly from rabbit to rabbit only by the intervention of blood-sucking arachnids. Potential vectors of this type were not encountered on continued inspections of the animals used.

Experiment BD 32 was an exception to other experiments in that the testis tissue cultures were maintained for 38 days. Myxoma virus was recoverable from fluids for only a week of this period, from 9-16 days after inoculation. One may speculate that the myxoma transformation had taken place in only a few cell areas. These areas may have been lost with the general deterioration of the tissue cultures which was observable after 2

weeks. Thus cells remaining would continue to produce fibroma virus as they had originally. Fibroma virus, and presumably myxoma, may grow in local areas surrounded by uninfected cells. Distribution of cytoplasmic inclusions in stained preparations of previous testis tissue cultures(5) give support to this hypothesis. The objective of continuing experiments on the Berry-Dedrick phenomenon is to increase the predictability with which it can be performed in tissue culture and to effect a chemical separation of the transforming factor contained in myxoma virus.

Summary. 1. Eleven experiments are described in which fibroma was transformed into myxoma virus in cultures of rabbit tissues, both of testes and of kidneys. 2. The transforming agent (TAM) was myxoma virus inactivated by heating at 65-67°C. 3. Live fibroma virus and TAM were inoculated into tissue cultures simultaneously and TAM was re-added at times of fluid change. 4. A good growth of supporting cells appeared essential to successful transformations.

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Effect of Insulin on Thyroid Gland Microhistometric Studies.* (23119)

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Several reports indicate that insulin treatment may affect the structure of the thyroid gland. The results of these investigations are somewhat contradictory: for instance, Watrin and Florentin(1) observed in guinea pigs, and Raiha and Uotila(2) in rabbits, a hypertrophy of the thyroid after administration of large doses of insulin. Raiha(3) and Schulze (4) produced in rabbits and in rats an involution of the thyroid by prolonged administration of insulin; but Schulze(5) himself found no changes in the thyroid of rats when one unit of insulin was given daily for 4 days and Borgorello(6) observed no histological changes in the thyroid of the rabbit, even after prolonged administration of insulin.

With these findings in view we used microhistometric methods to investigate the effect of insulin on structure of thyroid gland of rats exposed to room temperature (24-26°) and to cold (3°C).

Method and materials. Male rats of the Wistar strain, weighing 90 to 125 g were used. The diet, fed *ad libitum*, consisted of ground Purina laboratory chow, which contains according to our determinations, 200 μg of iodine/100 g. The rats received distilled water for drinking. Regular insulin (Lilly) was administered subcutaneously. The animals were kept in individual cages. One-half unit of insulin was given twice daily for 8 days. The controls received 0.1 cc saline subcutaneously at the same intervals. Higher doses of insulin were not used because the cold exposed animals succumbed on injection of slightly increased doses. At the end of the experiments the rats were sacrificed under ether anesthesia by exsanguination from the dorsal aorta. The thyroid glands were removed, dissected free of fat and connective

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TABLE I. Effect of Insulin on Rat Thyroid Epithelial Height (Mean Acinar Cell Height) at 24°-26°C, Exp. I; and at 2°-3°C, Exp. II.

Groups	No. of rats	Thyroid, MACH* (μ)
Exp. I—8 days, 24-26°C, *ad lib* feeding		
Saline, 0.1 cc sb b.i.d.	8	11.3 ± 0.06†
Insulin, 0.5 u sb b.i.d.	9	13.7 ± 0.3 (p 0.01)
Exp. II—8 days, 2-3°C, *ad lib* feeding		
Saline, 0.1 cc sb b.i.d.	8	14.7 ± 0.18
Insulin, 0.5 u sb b.i.d.	10	12.2 ± 0.15 (p 0.01)

* Mean acinar cell height. † Stand. dev.

tissue, weighed on Roller-Smith balance and transferred to small vessels containing 6 ml Bouin. The mean acinar cell height was measured in sections of the thyroid gland, according to the method of Starr and Rawson(7).

Results. In *Exp. I* the effect of insulin administration for 8 days in rats kept at room temperature (24-26°C) was investigated. Average mean acinar cell height of the thyroid was significantly elevated, 13.7 μ, when compared with the mean acinar cell height of 11.3 μ obtained in the controls. (Table I) (Fig. 1 and 2).

Exp. II. The effect of insulin administration was observed in animals placed in cold room (2-3°) for 8 days. Average mean acinar cell height of the thyroid after exposure to cold reveals less hypertrophy in insulin treated animals (12.2μ) than in the controls (14.7μ) (Table I).

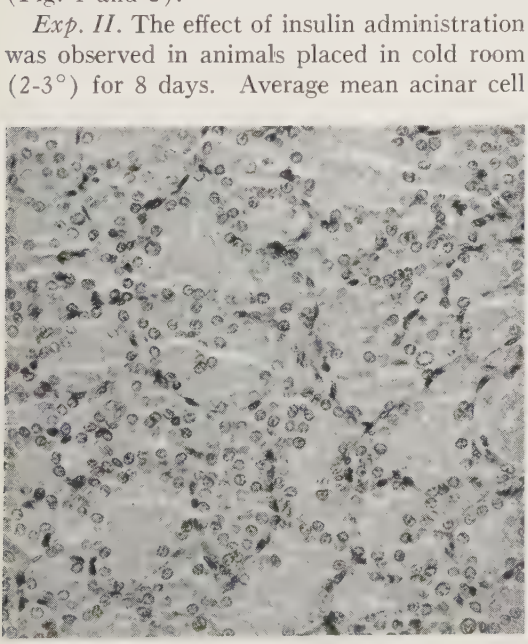

FIG. 1. Photomicrograph (×210) of thyroid gland of rats kept at 24-26°C after receiving insulin, 0.5 unit b.i.d. for 8 days. MACH, 13.7 μ.

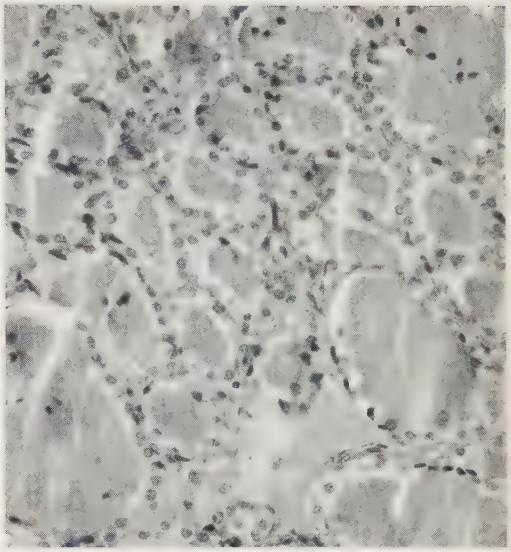

FIG. 2. Photomicrograph (×210) of thyroid gland in control rats kept at 24-26°C. MACH, 11.3 μ.

Discussion. Hypertrophy of the thyroid observed in the insulin treated animals at normal environmental temperature suggests hyperactivity of this gland and confirms the findings of Watrin and Florentin(1) and Raiha and Uotila(2). The mechanism of this hypertrophy may be explained either by the assumption that administration of insulin influences the thyroid by increased release of epinephrine(8), or that an increased release of corticoids is responsible for the hyperplasia of the thyroid, as shown in studies made by us(9) and by others(10,11).

The insulin effect observed in cold exposed animals, indicates that insulin decreases the usually observed hypertrophy of the thyroid. We do not have any explanation for this phenomenon. We assume, however, that there may be an indirect effect involving the pituitary adrenal system. Earlier experiments demonstrated that ACTH injections(9), similar to insulin, decreased the hyperplasia of the thyroid in cold.

Conclusions and summary. Wistar strain male adult rats weighing from 90 to 125 g received 0.5 unit of insulin twice a day for

8 days while at normal temperature (24-26°C), and in the cold room (3°C). At normal temperature the insulin-treated animals showed a significant increase in mean acinar cell height of the thyroid, giving a picture of hypertrophy of the thyroid gland. When the insulin treated animals were exposed to cold the thyroid glands showed a decrease in average mean acinar cell heights, as compared with the cold controls.

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Effect of Cobalt Administration on Myoglobin Content.* (23120)

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The mechanism of action of cobalt in production of polycythemia is not fully known. It is widely held that cobalt exerts some active stimulus to erythropoietic tissue, since a distinct reticulocytosis precedes the rise in erythrocyte count(1). Van Dyke *et al.*(2) have shown that the life span of erythrocytes is not increased in rats made polycythemic with cobalt. Hyperplasia of the bone marrow occurs(3) with little or no alteration in the total or differential leukocyte count(4).

The work of several investigators suggests that the action of cobalt is mediated through interference with some of the enzymes of cellular respiration(5,6). Such interference may induce a tissue anoxia which in turn elicits compensatory erythrocytosis. If this is the case, myoglobin, which lies intermediate in the chain of respiratory events between the erythrocytes and the respiratory enzymes(7), might be expected to increase in amount so as to augment oxygen transfer. The present study was made to determine the effect of cobalt on myoglobin concentration.

Methods. Twenty-four Sprague-Dawley female rats weighing 118 to 144 g were divided into 2 groups of 6 pairs each. One member of each pair was randomly chosen to be treated while its mate served as the control. The animals were individually caged in air-conditioned quarters and were pair-fed on Purina laboratory meal. Water was available *ad libitum*. Cobalt chloride hexahydrate in aqueous solution was administered by subcutaneous injection. The 6 treated animals of Group 1 received daily 2.5 mg/kg body weight of the compound as a 0.25% solution and those in Group 2 received 5.0 mg/kg body weight as a 0.5% solution. All control animals were injected with a comparable volume of demineralized water. Hematological examinations were made weekly on tail blood. Erythrocyte and leukocyte counts were done by standard technics; hemoglobin was measured as oxyhemoglobin. The packed cell volume was determined by the micromethod of Guest and Siler(8).

At the end of 88 days all animals were sac-

* The material presented here is taken from thesis submitted by Willard R. Faulkner in partial fulfillment of requirements for Ph.D. degree from Vanderbilt University.

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TABLE I. Mean Initial and Terminal Hemoglobin Concentrations.

Level of cobalt, mg/kg	Treatment, days	Treated		Controls	
		Initial	Terminal	Initial	Terminal
		g/100 ml ± S.E.			
2.5	80	13.6 ± .3	21.2 ± .2	14.2 ± .4	15.9 ± .1
5.0	86	13.5 ± .3	21.3 ± .2	14.2 ± .5	15.5 ± .2

Six rats in each group.

rificed by decapitation. Myoglobin analyses were performed on the combined gastrocnemius and soleus muscles from each leg by a modification of the method of Poel(9).

Results. The degree of polycythemia which had developed at termination of the experiment is shown in Table I. Erythrocyte counts and packed cell volumes paralleled hemoglobin values closely throughout the experiment. The data in Table II compare myoglobin concentration of the treated and control animals at time of sacrifice and show that the amount present was not altered by treatment with cobalt.

TABLE II. Effect of Cobalt on Myoglobin.

Level of cobalt, mg/kg	Treatment, days	Myoglobin	
		Treated	Controls
		mg/g ± S.E.	
2.5	88	1.26 ± .03	1.25 ± .05
5.0	88	1.22 ± .03	1.17 ± .02

Six rats in each group.

Summary. Administration of cobalt to rats at a level sufficient to produce a marked polycythemia did not alter myoglobin concentration.

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A Double Yoke Harness for Prolonged Immobilization of Small Animals.* (23121)

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Prolonged immobilization of a small animal is often required either for collecting products of metabolism or for continuous administration of solutions, drugs, blood etc. For these purposes technics have been developed(1,2,3) by which the animal is almost completely immobilized. However, extensive and prolonged muscular inactivity may lead to death. Movement is obviously essential for the physiologic processes of the organism. Motivated by these considerations we have developed the following method for immobilization of continuously infused rats, using a metal harness consisting of a double yoke.

Methods. The double yoke is formed of 2 galvanized iron plates having a large eccentrically placed hole (Fig. 1), through which the rat's body is passed. A stainless steel wire (W, Fig. 1) is welded along the inner edge of the hole to blunt it. G-clamps (Cl, Fig. 1)

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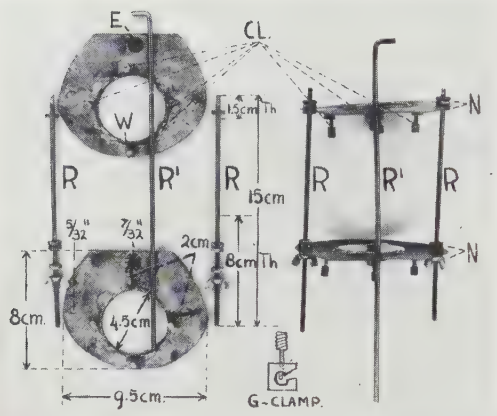

FIG. 1. Double-yoke harness. E = Eyelet for iron rod (R^1). CL = G-clamps. W = Steel wire. R = Brass rod. Th = Thread on brass rod. N = Nut.

with knurled screws are welded at the 4 quadrantal points of each metal plate, on the hind side. The yokes are interconnected by 2 threaded metal rods (R, Fig. 1) which permit adjustment of the distance between the 2 plates. Each of them passes through the side holes of the plates and is held in position by means of 3 nuts (N, Fig. 1). An eyelet (E, Fig. 1) at the top of each yoke is designed for the passage of a metal rod (R^1, Fig. 1 & 2) of the desired length, on which the yokes are suspended. They slide along this rod when the animal moves back and forth.

Technic. The rat (200-250 g body wt) is anesthetized and inch-wide strips of hair clipped around the body, one behind the forelegs and the other in front of the hind-legs. The skin is cleaned with 70% alcohol. The plates are fastened to the rat's body by a silver wire (#27) with which the skin is undersewn in large (¾") stitches. The parts of the stitches that overlay the skin are only ¼" long. They are fastened into the G-clamps (CL, Fig. 1) on the back of the plates by their knurled screws. The raised skin folds are arranged to almost close off the large holes of the yoke plates. The wire ends are then twisted and care is taken not to encircle too tightly the rat's thorax and abdomen, in order to avoid restriction of its respiratory movements. The iron rod (R^1, Fig. 1 & 2) is then passed through the eyelets (E, Fig. 1) and suspended on a rod consisting of 2 horizontal iron bars (B, & B_2 Fig. 2) which may hold several rats in position letting their feet touch the wired bottom (Fig. 2). The rats can walk backward and forward the few paces allowed by the yoke sliding on the iron rod, and can reach their food (F & Wa, Fig. 2) with ease. However the front yoke makes it impossible for the rat to turn its head and bite through the cannula (T, Fig. 2) carrying the infusion fluid.

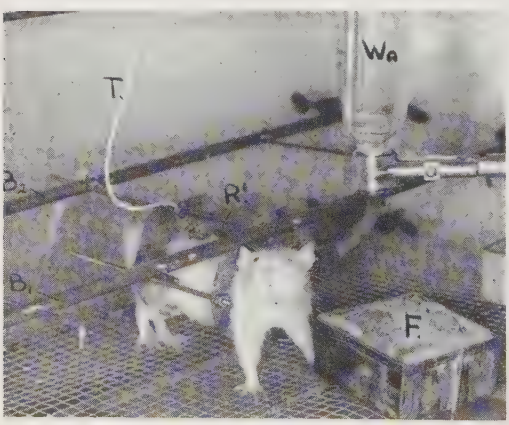

FIG. 2. Rat wearing harness. B_1; B_2 = Iron bars. T = Tubing carrying infusion fluid. R^1 = Iron rod. F = Food. Wa = Water bottle.

Results. 25 rats were immobilized by the double yoke harness and 22 of them had a continuous glucose infusion into the portal vein for 7 to 15 days, when they were freed of their yokes. The other 3 rats were not infused but were immobilized for 5 to 6 weeks. All animals appeared surprisingly healthy and clean at the end of their immobilization period.

Summary. A method has been devised for immobilization of small animals in a metal double yoke harness to carry out prolonged infusions. The harness is well tolerated and permits a degree of movement necessary for the well being of the animal.

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Effect of Plasmapheresis on Blood Cholesterol Levels in the Dog.*† (23122)

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The hypercholesterolemia of nephrotic syndrome is regularly associated with heavy proteinuria and diminished plasma albumin concentration. Clinically, proteinuria precedes alterations in plasma cholesterol and albumin levels. Recently, Rosenman and his co-workers(1) have presented evidence indicating that plasma cholesterol elevation in nephrotic rats occurs as a consequence of heavy proteinuria and subsequent plasma albumin depletion. We have reasoned that if hypercholesterolemia is a result of plasma protein depletion, it should occur if the plasma proteins are lost via a diseased kidney, or by some other route in the presence of substantially normal renal function. In the present experiments the levels of blood cholesterol have been studied following depletion of plasma protein by the technic of plasmapheresis.

Methods. Adult male dogs were used in all experiments. The animals were trained to lie on their backs with legs restrained. A 16-gauge needle was inserted into the external jugular vein and the effluent blood was collected in a sterile vacuum flask containing heparin. One hundred fifty to 300 ml of blood was collected daily, Monday through Friday each week. Immediately after collecting the blood, its temperature was lowered to 2°C. The erythrocytes were separated from plasma by centrifugation in the cold and were washed once with cold sterile 0.89% sodium chloride solution. The washed erythrocytes were then suspended in 0.89% sodium chloride solution equal to the volume of plasma removed and were kept in the cold until reinfused into the dog from which they were taken. The warmed, washed erythrocytes taken the day before were given intravenously through the needle in the jugular vein after the day's bleeding was completed. The animals tolerated this procedure without noticeable ill effects. If the hematocrit began to fall, additional washed erythrocytes from a donor animal were given. Animals were weighed daily, and weight was maintained as closely as possible by increasing food intake. All glassware and tubing were kept sterile. Rectal temperatures were taken daily and did not deviate from the normal range. *Diet*: Animals were initially given a protein free 7% fat diet of the following composition in the form of a baked cake: corn starch 38.6%, sucrose 19.3%, crisco 12.9%, dextrose 6.4%, corn oil 2.4%, baking powder 1.6%, salt mixture(2) 1.2%, bone ash 4%, kaolin 4%, water 9.6%. After one week, 0.5 g/kilo body weight of protein (horsemeat) was added and the animals were continued on this diet for duration of experiment. Vitamin supplements were added to the diet daily. *Chemical determinations*: Plasma albumin and globulins were determined by Kingsley's Method(3). Cholesterol was determined by modification of the method of Schoenheimer and Sperry (4).

Results. Results are shown in Fig. 1 (A-D). The calculated regression line is indicated on each plot as is the correlation coefficient "R". The significance of the correlation coefficient has been calculated according to the formulae: $Z = 1.1513 \times \log_{10} \frac{1+r}{1-r}$. Standard deviation of Z = Fishers "t" = $\frac{1}{\sqrt{N-3}}$ where N = number of paired observations. P values are obtained by reference to Fishers Table of t, where t has an infinite number of degrees of freedom. Thus, for dog A, P = 0.1, for dog B $<$ 0.01, for dog C 0.02 – 0.01 and for dog D $<$ 0.001. This indicates a

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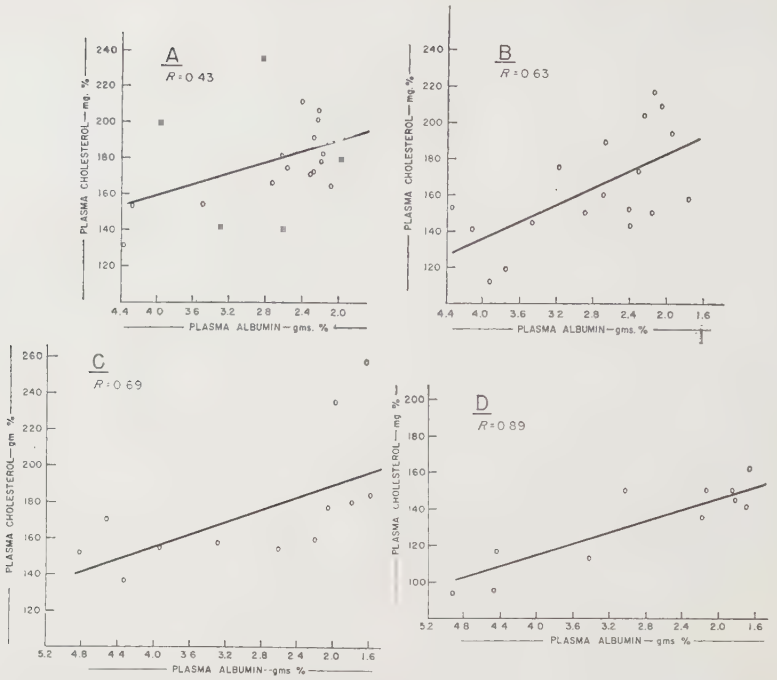

FIG. 1. Relation between plasma cholesterol and plasma albumin during plasmapheresis in each of 4 separate animals. R = Correlation coefficient.

satisfactory correlation of the plotted data from dogs B, C and D, but not from dog A. Inspection of Fig. 1-A indicates that a single determination that departs radically from the remainder (at plasma albumin level of 4) keeps this plot from attaining a greater degree of significance.

It is apparent, that as the plasma albumin concentration is experimentally reduced, the plasma cholesterol concentration rises. Except for Fig. 1-D, a curve with increasing slope at lower plasma albumin levels would better fit the experimental observations. It may be that significant increases in plasma cholesterol do not occur until the plasma albumin falls below a concentration of approximately 2.5 g %. It is also possible that the increased rise in plasma cholesterol at lower albumin levels is related to some factor not accounted for in these plots, such as time. There may be a lag in the maximal elevation of plasma cholesterol in response to a given level of reduced plasma albumin.

Total plasma globulin concentrations were not significantly altered during the experiments. Two control dogs were fed the same diet as the experimental animals, but they were not plasmapheresed. Although their plasma albumin concentrations fell from levels of about 4.5 g % to approximately 3.5 g %, no consistent alteration was observed in the levels of plasma cholesterol.

Discussion. It is possible that chronic plasmapheresis depletes the animal of a variety of substances only a few of which may have been measured in these studies. However from the data at hand, the rise in plasma cholesterol that occurs following plasmapheresis appears to depend on depletion of plasma albumin. Total plasma globulin concentrations were not significantly altered by plasmapheresis. The animals were kept at nearly constant weight, and great care was exercised in maintaining erythrocyte counts and hemoglobin concentrations at normal levels.

These data support the earlier findings of Boggs and Martin(5) and Fishberg and Fishberg(6) and recent observations of Rosenman, Friedman and Byers(1). Furthermore, it is demonstrated that albumin loss need not occur through a diseased kidney to initiate elevations in plasma cholesterol. If sufficient

plasma albumin is lost from the animal to deplete plasma albumin concentration, elevation of plasma cholesterol will occur whether the protein is lost via a heavy proteinuria, or whether it is withdrawn directly from the blood stream in the presence of substantially normal kidneys. The corollary to this experiment has already been performed in the rat by Rosenman *et al.*(1) when they demonstrated that plasma cholesterol values fell toward normal following the infusion of bovine albumin into nephrotic animals.

Summary. Chronic plasmapheresis in the dog resulted in a fall in plasma albumin and an elevation of plasma cholesterol concentration.

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Boundary Electrophoresis of Human Parotid Saliva. (23123)

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The ultraviolet spectrum of human parotid saliva has been shown to possess bands characteristic for protein(1). Recently, Pigman *et al.*(2) stated that parotid saliva contained 8 electrophoretic components with a major peak of low mobility at pH 8.6. The present study presents electrophoretic patterns of 6 specimens of parotid saliva from 5 individuals.

Methods. Six samples of parotid saliva of 60 ml each were collected from 5 individuals by means of a modified* Lashley cup (3). They were concentrtaed by ultrafiltration(4) at 4°C, dialyzed against 0.1 ionic strength veronal at pH 8.6, filtered, made up to 11 ml, and subjected to boundary electrophoresis in the standard cell of the Aminco Model B Electrophoresis Apparatus. Duplicate specimens of 60 ml of parotid saliva were concentrated(4), made up to 20 ml and samples were taken before and after ultrafiltration for protein analysis by the biuret reaction(5). Mobilities were determined according to standard procedures(6) and the relative concentrations were calculated from the Rayleigh fringes(7) shown in Fig. 1 with the customary assumption that the refractive index increment of all the components was the same. The minor components II and IV (Fig. 1) were included with component III in the calculation of the relative concentrations.

Results. As shown in Table I, the extent of concentration of protein after ultrafiltration (5.4 fold) closely paralleled concentration in volume (5.5 fold, or 60 ml to 11 ml), indicating that essentially no protein was lost by ultrafiltration. Before ultrafiltration the parotid salivas contained 18.7-58.9 mg %

TABLE I. Protein Content of Parotid Saliva before and after Ultrafiltration (mg %).

Subject	Before	After	Degree of conc. Volume	Protein
A	41.0	243.5	5.5	5.9
B	52.0	269.2	"	5.2
C	18.7	98.3	"	5.3
D	51.5	259.4	"	5.0
E	58.9	323.8	"	5.5
		Mean	5.5	5.4

* Prepared in Instrument Shop, N.I.H.

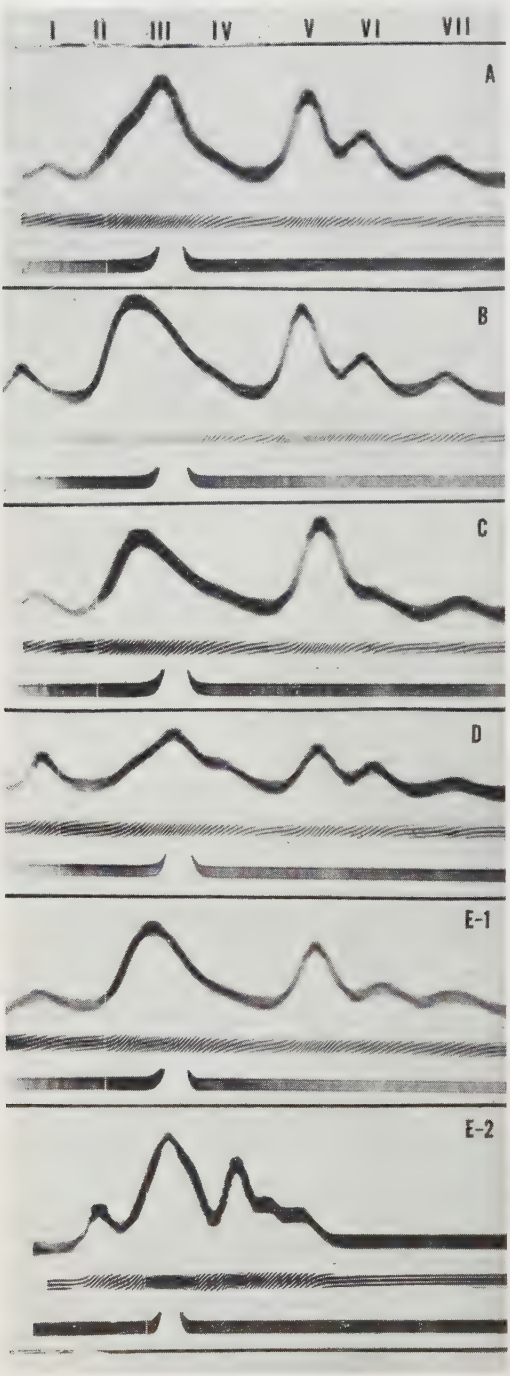

FIG. 1. Electrophoretic patterns of 6 samples of concentrated (× 5.4) human parotid saliva from 5 individuals. Time of run 7200 sec. for samples A to E-1; for samples E-2, 3600 sec. Potential quotient 6.25 volts/cm. Starting boundary and Rayleigh fringes are included with each schlieren electrophoretic pattern.

protein.† The concentrated parotid saliva submitted for electrophoresis contained 98.3 – 323.8 mg % protein, and both positively and negatively charged components were observed.

As shown in Table II and Fig. 1, the mobilities and the relative concentration of the components were essentially similar for all individuals. To date, no electrophoretic patterns on parotid saliva have been presented in the literature although a preliminary report has appeared stating that 8 components have been observed with a major fraction showing a very low mobility at pH 8.6(2). Reports have also appeared on the electrophoresis of whole human saliva(9,10).

In the present study, 5 distinct peaks (I, III, V, VI, VII) were seen in every case, and 2 additional peaks (II, IV) appeared as "shoulders" in some individuals. Two peaks (III, V) accounted for 75% of the components with the major peak comprising 50% of the fractions and showing a mean mobility of $+0.3 \times 10^{-5}$ cm^2 sec.$^{-1}$ volt^{-1}. This finding confirms the report of a peak of similar mobility. The fastest moving component had a mean mobility of -4.18×10^{-5} cm^2 sec.$^{-1}$ volt^{-1}. Although identification of the various components awaits further investigation, none has the mobility of albumins and some may perhaps be globulins in view of their low mobility(4,6). Since no runs were made at pH values other than 8.6, the extent of the contribution of the salt anomaly to peaks II, III, and IV was not evaluated.

Summary. Six samples of parotid saliva from 5 individuals were subjected to boundary electrophoresis at pH 8.6 in 0.1 ionic strength veronal buffer. Seven peaks were obtained with 2 peaks accounting for 75% of the components. The major peak was essentially stationary with mean mobility of $+0.3 \times 10^{-5}$ cm^2 sec.$^{-1}$ volt^{-1}, and comprised 50% of the components. The fastest moving constituent had a mean mobility of -4.18×10^{-5}

† These findings do not agree with those of Bramkamp who reported concentrations varying from 10-90 mg % protein nitrogen(8) or approximately 60-560 mg % protein. Variance is probably due to differences in analytical procedures.

TABLE II. Fractions of Concentrated Parotid Saliva Separated by Boundary Electrophoresis.*

Component	A	B	C	D	E-1†	E-2†	Mean ± S.E.
			Mobility $\times 10^{-5}$ cm^2 sec.$^{-1}$ volt^{-1}				
I	+1.90	+2.24	+2.11	+2.28	+2.14	+2.15	+2.14 ± .08
II	+ .85	+ .60	+ .86				+ .77 ± .08
III	+ .17	+ .24	+ .57	+ .50	+ .39	+ .04	+ .32 ± .08
IV	− .74	− .60	− .83	− .81	− .71	− .93	− .77 ± .08
V	−2.11	−1.94	−2.22	−1.99	−2.22	−2.02	−2.08 ± .05
VI	−3.02	−2.84	−3.07	−2.91	−3.31	−2.95	−3.02 ± .07
VII	−4.25	−4.05	−4.20	−4.21	−4.49	−3.86	−4.18 ± .09
			Relative concentration (%)				
I	6.6	13.2	8.0	7.4	8.0	5.6	8.1 ± .93
II, III, IV	50.8	52.8	50.0	53.3	52.0	51.4	51.7 ± .51
V	21.3	20.8	32.0	20.5	22.7	23.6	23.5 ± 1.77
VI	16.4	7.5	6.0	14.7	8.0	9.7	10.4 ± 1.72
VII	4.9	5.7	4.0	4.1	9.3	9.7	6.3 ± 1.05

* Taken from descending boundary. † Same individual run 3 wk apart.

cm^2 sec.$^{-1}$ volt $^{-1}$.

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Paper Chromatographic Method for Estimation of Phenylalanine.* (23124)

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In phenylketonuria serum phenylalanine levels are high(1). Removal of phenylalanine from the diet results in a marked decrease in blood phenylalanine to near normal levels(2,3). The methods for determination of phenylalanine are largely based on modifications of the Kapeller-Adler procedure(4) in which phenylalanine is oxidized to benzoic acid and subsequently nitrated to the 3,4-dinitro derivative(3,5). The enzymatic decarboxylation of phenylalanine to phenylethylamine and determination of the resulting base as described by Udenfriend and Cooper has also served as a method for obtaining phenylalanine values in serum(6). When children are to be treated with a phenylalanine-low diet, there is need for a simple and rapid method for determination of serum phenylalanine. We have used a paper chromatographic method requiring a very small volume of blood in order to obtain a clear picture of the individual response to a low phenylalanine diet.

Methods. Phenylalanine in blood serum. A small sample of blood such as can be obtained from skin puncture is sufficient for the estimation of the phenylalanine content of serum. Protein is removed by adding 2 ml

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of 95% ethyl alcohol to 0.5 ml of serum. The precipitated protein is removed by centrifugation or suction filtration. The filtrate, a 5-fold dilution of the original sample, is used directly for chromatography. Aqueous solutions of dl-phenylalanine containing 0.2, 0.3, and 0.4 mg/ml are prepared for use as standards. Sheets of Whatman No. 1 filter paper, 7 in. x 11 in., are marked along a line 2 cm from the long edge of the paper at intervals of 2 cm. Along this line the standard solutions are applied in increasing amounts on positions 1, 3, 5. Positions 11, 9, 7, are duplicates of 1, 3, 5, respectively. The solutions are applied with 5 μl capillary pipettes obtainable from Microchemical Specialties Co., Berkeley, Calif. The ethanol filtrate of the unknown serum is applied in 5 μl increments until volumes of 50, 75, and 100 μl have been added to spots 2, 4, and 6. Spots 8, 10, and 12 are duplicates of 2, 4, and 6. These increasing amounts are applied to the paper by pipetting a 5 μl volume on a spot several times, allowing the spot to dry each time, until the total volume has been added to the spot. This time-consuming method of application cannot be avoided, since the spot diameter must be kept small and should not exceed 12 mm. The sheets should be prepared in duplicate. If only a very small volume of blood is available 0.2 ml of serum and 0.8 ml of ethanol give sufficient filtrate to prepare a single sheet. When the sample and standard spots have dried, the sheets are rolled into a cylinder, and the short edges are stapled together with great care that the edges do not touch after stapling. Excessive handling of the sheets must be avoided to prevent contamination from ninhydrin-reacting components of sweat. These cylinders are placed upright in a solvent mixture, prepared by mixing 70 ml n-butyl alcohol (technical grade), 20 ml 95% ethyl alcohol, and 20 ml water, in a Pyrex jar of 10 to 18 in. height, approximately 8 in. in diameter. Airtight closure is provided by a glass plate to cover the jar. The depth of solvent should be such that the bottom edge of the cylinder is covered to within ½ in. of the line along which the spots were applied. The solvent mixture may be used for several chromatograms prepared at different times provided the container is always kept tightly closed. If the solvent composition is altered by evaporation, phenylalanine will not be separated from other serum constituents. Chromatographic equipment can readily be improvised. As an alternative to Pyrex jars, stone crocks are perfectly suitable as chromatogram chambers. Since the bottoms are usually uneven, the solvent should be placed in a Pyrex pie plate obtained from a hardware or variety store, or in a Petri dish. The sheets should remain in the solvent mixture about 6 hours. The sheets are then removed and dried in air at room temperature at least two hours before further treatment. The dried sheets are sprayed with a reagent prepared by adding 200 mg ninhydrin to 85 ml butyl alcohol, 10 ml water, 5 ml 95% ethanol. The sheets are heated in an oven at 90°C for 10 minutes.

Results. Phenylalanine appears as a blue spot at Rf .42. (Rf is the ratio of the distance the phenylalanine has traveled to the total distance the solvent has traveled.) Other amino acids are characteristically purple in color and migrate to different positions. Phenylalanine content of the serum can be estimated by visual comparison with the standard spots. A more precise determination can be made with a densitometer, if available. Density measurements (1 – log % transmission) when plotted *vs.* micrograms of phenylalanine give a straight line in the range 0.5 – 2.5 μg of phenylalanine.

When the serum phenylalanine concentration is below 5 mg %, spots containing 0.5 μg of phenylalanine standard may replace the 2 μg phenylalanine standard to extend the concentration range downward to 2.5 mg %, corresponding to the concentration found in normal serum. The concentration range can be extended upward by using a smaller volume of diluted serum. For example, 15, 25 and 50 μl of serum filtrate should be used in examining blood from untreated phenylketonurics. As mentioned earlier, greater accuracy is possible if densitometric measurements can be made, but for clinical guidance of children with phenylpyruvic oligophrenia treated with a phenylalanine-free diet the changes in serum phenylalanine content are

TABLE I. Serum Phenylalanine Levels in a Phenylketonuric Child.

	Date	Phenylalanine (mg %)
Regular diet	4/ 3/56	34
	5	45
	7	24
	9	47
	11	24
Low phenylalanine diet (begun 4/12/56)	4/14/56	18
	16	15
	18	11
	19	9.5
	24	4.3
	31	4.9
	5/ 2	5.3

so marked that the more exact measurements are not needed. Table I gives values for serum phenylalanine in a phenylketonuric child several days before and after institution of treatment with a phenylalanine-low diet.

In our experience phenylalanine in serum from phenylketonuric children on a regular diet may vary from concentrations of 20 mg% to 50 mg%. When phenylalanine has been removed from their diet, the serum levels gradually decrease to a minimum value between 2 and 4 mg%. The blood levels generally found among the phenylketonuric children on diets low in phenylalanine are usually between 4 and 8 mg%.

Data listed in Table II show recoveries of phenylalanine added to serum. The determinations were made as described above, using a Model 525 Photovolt Densitometer. The range of recovery was from 80% to 106%, with an average recovery of 95%.

Phenylalanine in urine. When urinary excretion of phenylalanine is high relative to other urinary amino acids the method described for serum phenylalanine can be applied to urine, using duplicate spots of 5 μl of $\frac{1}{4}$ dilution on spots 2 and 8, 5 μl of $\frac{1}{2}$ dilution on spots 4 and 10, and 5 μl spots of undiluted urine on positions 6 and 12. Urinary levels from 0.10 mg/ml to 1.6 mg/ml may be determined.

TABLE II. Recovery of Phenylalanine Added to Serum.

Sample No.	Phenylalanine			
	Added, μg	Found, μg	Recovered, μg	%
1	0	69		
	400	454	385	96
	200	228	159	80
2	0	0		
	200	204	204	102
3	0	22		
	50	75	53	106
4	0	95		
	50	138	43	86
5	0	71		
	100	172	101	101

Phenylalanine in cerebrospinal fluid. Phenylalanine levels may be determined on untreated cerebrospinal fluid using the technic described for serum. Neither deproteinization nor desalting is required. Duplicate spots of 20, 50, and 100 μl are used. The concentration range covered using these volumes is from 0.5 mg% to 10 mg%. The average value for untreated phenylpyruvic oligophrenic children is 8 mg%. Phenylalanine concentration in the spinal fluid falls below 0.5 mg% after a period on phenylalanine-free diet. Glutamine is generally the only other ninhydrin-reacting substance visible in 100 μl of spinal fluid.

Summary. For paper chromatographic estimation of phenylalanine, 0.5 ml samples of blood serum are deproteinized with 4 volumes of 95% ethyl alcohol and the filtrate used for preparation of the chromatogram. The method is especially useful in study of phenylketonuric infants, and in following the changes in serum phenylalanine when a low-phenylalanine diet is used for their treatment. The recovery range of phenylalanine added to serum was from 80% to 106% with an average recovery of 95%.

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Blood Glutathione in Mild Diabetes Mellitus Before Treatment and During Sulfonylurea-Induced Hypoglycemia.* (23125)

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To ascertain whether arylsulfonylurea compounds influence blood glutathione concentrations, determinations of reduced glutathione were made following oral administration of tolbutamide (Orinase)† or carbutamide (BZ-55)† to patients with normal carbohydrate metabolism and patients with mild diabetes mellitus. Glutathione values were much lower in the diabetic patients both before and after administration of the sulfonylureas. However, no change in glutathione levels was observed in either group during the acute hypoglycemic response to these compounds, nor in diabetic patients who received therapeutic doses of tolbutamide for several days.

Material and methods. The patients were all men. Each of 9 control patients had a normal oral glucose tolerance test. Twelve of the 15 diabetic patients were eating at least 200 g of carbohydrate daily, while the others received 150 g daily. Insulin had never been given to 12 of the diabetics, and had been discontinued in the others at least one week before the test. The ages of control patients ranged from 24 to 62 years, with a mean of 37 years. The diabetic patients were older, from 46 to 68 years of age with a mean of 59 years, since most of them were selected for probable susceptibility to sulfonylureas. Liver function tests were normal in all 24 individuals. Following an overnight fast 3 g of tolbutamide were given orally to all control patients and to 11 of those with diabetes. The other 4 diabetics received 3 g of carbutamide orally. Oxalated specimens of venous blood were obtained before administration of sulfonylurea and at hourly intervals for 5 hours. Duplicate determinations on whole blood were done for glucose by the Somogyi-Nelson method(1), and for reduced glutathione by the method of Woodward and Fry (2). Hematocrits obtained at each interval on heparinized blood enabled the blood glutathione concentration to be expressed in terms of 100 cc of packed red blood cells.

Results. In analyzing the findings the diabetic patients are discussed as a group because, regardless of magnitude of individual hypoglycemic response or sulfonylurea agent administered, the glutathione data yielded by each diabetic were entirely similar.

In Table I the desultory hypoglycemic response to tolbutamide in the control group contrasts sharply with the marked depression of the blood glucose level induced in most of the diabetic patients by tolbutamide or carbutamide. The average maximum depression from the fasting glucose value was 14% in the former group and 43% in the latter.

However, in neither group was the fall in blood glucose accompanied by an important change in blood concentration of reduced glutathione (Table I). In control patients the glutathione level remained essentially constant throughout the test period. In the diabetic group the mean 5-hour glutathione value of 70.5 $\pm$ 7.6 (S.D.) mg/100 cc of packed red blood cells was insignificantly higher ($p = 0.1$) than the mean fasting concentration of 66.6 $\pm$ 5.6.

At the same time, the actual blood glutathione concentration of diabetic patients was distinctly subnormal. The difference between the mean fasting blood glutathione concentration in the normal group (81.3 $\pm$ 6.7 mg/100 cc of packed red blood cells) and the respective value in the patients with diabetes (66.6 $\pm$ 5.6 mg/100 cc of packed red cells) represents a significance of $p = <.001$ (Table I). Equally significant differences between respective values persisted throughout the test period except at the 1-hour interval, where $p = <.01$.

* This study was supported in part by research grant from U. S. Public Health Service.

† Tolbutamide was kindly supplied by Upjohn Co., and carbutamide by Eli Lilly and Co.

TABLE I. Blood Concentrations of Glucose and Reduced Glutathione after Administration of 3 g of Tolbutamide or Carbutamide to Non-Diabetic and Diabetic Patients.

Patients	Fasting	1 hr	2 hr	3 hr	4 hr	5 hr	Max fall from fasting level
	Blood glucose (mg %—Somogyi)						
Non-diabetics (9)*	77 ± 4†	75 ± 4	72 ± 4	72 ± 5	68 ± 5	68 ± 4	14 ± 6%
Diabetics (15)	177 ± 59	167 ± 62	149 ± 60	127 ± 56	115 ± 57	107 ± 51	43 ± 11%
	Reduced blood glutathione (mg/100 cc packed red blood cells)						
Non-diabetics (9)	81.3 ± 6.7	78.6 ± 8.5	81.9 ± 9.1	81.0 ± 7.6	81.4 ± 7.5	83.1 ± 7.5	
Diabetics (15)	66.6 ± 5.6	67.9 ± 4.9	67.3 ± 5.3	68.1 ± 7.4	69.3 ± 6.1	70.5 ± 7.6	
p‡	<.001	<.01	<.001	<.001	<.001	<.001	

* No. of patients. † Stand. deviation. ‡ Derived from comparison of respective glutathione values at each interval.

Blood glutathione concentrations were determined in 6 diabetic patients who received therapeutic doses of tolbutamide for 6 days or more. Table II demonstrates that the low pre-treatment glutathione values remained unchanged whether or not the drug was effective in restoring normal fasting blood sugar levels.

Discussion. The strikingly subnormal blood glutathione concentrations observed in these diabetic patients disagrees pointedly with recent reports(3,4) of normal glutathione values in such patients. Two major considerations may account for this apparent discrepancy.

Woodward and Fry(2) originally expressed concentration of reduced blood glutathione in terms of mg/100 cc of whole blood. Since blood glutathione exists entirely within the erythrocyte, however, it has subsequently been emphasized(5,6) that precision requires its expression in terms of red cell mass, the procedure used in the present study. In reports employing the former and less accurate means of presenting glutathione concentrations in diabetic patients(3,4) blood glutathione levels which are actually depressed may have appeared normal when masked by the hemoconcentration accompanying severe glycosuria.

TABLE II. Persistence of Subnormal Blood Glutathione Concentrations after Several Days of Tolbutamide Therapy in Patients with Diabetes.

Patient	Fasting blood glucose (mg % –Somogyi)	Blood glutathione (mg/100 cc packed RBC)	Daily tolbutamide dosage and duration of treatment
1	329	62.4	P.t.*
	281	57.8	5 g for 6 days
2	152	60.8	P.t.
	148	62.5	4 g for 2 mo
3	142	67.4	P.t.
	68	61.5	5 g for 6 days
4	139	72.6	P.t.
	87	70.8	4 g for 6 days
5	132	69.3	P.t.
	80	65.3	5 g for 7 days
6	113	74.0	P.t.
	82	70.9	4 g for 8 days

* P.t. = Pretreatment.

The opinion that blood glutathione concentrations are normal in diabetes has also been propagated by the tendency of most workers to consider their diabetic populations as homogeneous groups. Such an assumption ignores the possibility that glutathione concentration bears a relationship to degree of diabetic control. However, experimental(7) and clinical studies(8,9) strongly suggest that normal glutathione metabolism, in both diabetic and non-diabetic patients, requires the existence of normal carbohydrate tolerance. Although blood levels of glutathione are known to be low in frank ketosis(10), no analysis has yet appeared of glutathione concentrations in controlled compared to uncontrolled but non-ketotic diabetics. In the present study each diabetic patient exhibited an elevated fasting blood sugar as evidence of mild to moderate uncontrol of his disease, but none had ketosis.

Conversely, factors which are known to be associated with true depression of the blood glutathione concentration were not present in these diabetic patients. Such factors include starvation, protein depletion, liver disease and

ketosis(6).

The insignificant rise in blood glutathione concentrations coincident with sharply falling blood glucose levels in the diabetic patients indicates that this convenient potential index of intracellular sulfhydryl activity is unaffected by the arylsulfonylurea compounds. Nevertheless, the essentially unchanged glutathione values do not entirely erase the possibility that metabolic activity of these agents may be mediated by altering the intracellular availability of sulfhydryl groups. Experimental work(11) demonstrates that profound changes in tissue concentrations of glutathione may or may not be accompanied by variations in the blood glutathione level. A similar situation may also exist in man.

Summary. 1. Blood glucose and reduced glutathione determinations were performed at intervals on 9 non-diabetic patients and on 15 patients with moderately uncontrolled diabetes following oral administration of 3 g of tolbutamide or carbutamide. 2. Mean maximal decrease from fasting blood glucose value was 14% in patients with normal carbohydrate metabolism, and 43% in the diabetic group. 3. In control patients there was no change in glutathione levels during the test period. In diabetic patients a minimal, insignificant rise had occurred 5 hours after ingestion of the hypoglycemic agent. 4. The mean blood reduced glutathione concentration was strikingly lower in patients with diabetes than in those whose carbohydrate metabolism was intact. In 4 patients subnormal values persisted despite restoration of normal fasting blood glucose levels by daily therapeutic doses of tolbutamide.

The technical assistance of Melba Loveall, Lora Keller and Mildred Brennan is gratefully acknowledged.

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Human Chorion Cells: Cultivation and Susceptibility to Viruses.* (23126)

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The ready availability and usefulness of amnion cells in tissue culture virology have been demonstrated since the original publication of Zitcer *et al.*(1). Modifications of the amnion cell culture, virus spectrum, and application to primary isolation have been reported by several groups(2,3). The chorion is a placental by-product of every amnion trypsinization, and therefore the usefulness of the chorion was explored.

Materials and methods. Chorion cell cultures. The methods employed were essentially similar to procedures used for the amnion(2). Fresh membranes were collected within one

* The authors wish to thank the staff of the Obstetrical Service of the Natl. Naval Med. Center, Bethesda, whose continued cooperation made this study possible.

hour after delivery into Mason jars containing non-refrigerated Hanks' balanced salt solution (BSS) with antibiotics in concentrations described below. The chorion was separated from the amnion with forceps, and washed 4-6 times in Hanks' BSS. This procedure revealed a relatively blood-free membrane 2-3 times the thickness of the amnion, smooth and pink on one side and rough, yellow and opaque on the other. The tissue was then cut into approximately one cm squares for digestion in 0.25% trypsin and Hanks' BSS while kept in suspension by a magnetic stirrer. After one hour, supernatant fluid was discarded; the chorion was minced finely, and re-exposed to fresh trypsin solution at room temperature for 12-20 hours. The resulting supernatant which contained dispersed chorion cells was filtered through gauze and centrifuged for 10 minutes at 1200 rpm in a horizontal International Size 2 centrifuge. The cell pack was then washed twice in tissue culture medium with approximately 5% calf serum. One membrane generally yielded 1.0-1.5 ml cell pack, which was dispersed 1:125 in Eagle's basal medium(4) with 10% horse serum heated at 56°C for 30 minutes. Five-tenths ml amounts were dispensed into each stationary culture tube and incubated at 37°C. After cell attachment at 48 hours, the nutrient medium was replenished. A cell sheet generally formed in 4-6 days and the horse serum was reduced to 4%. Cultures were changed with 1 ml fluid once a week and could be maintained for 30 days. All fluids used contained 100 units penicillin, 100 μg achromycin, 50 units mycostatin, and 100 μg streptomycin/ml. *Chorion susceptibility to viruses.* Various viruses, usually prototypes of standard strains in use at the NIH, were employed throughout. One tenth ml of each virus pool was inoculated into 2 chorion tubes. These cultures were observed daily for appearance of maximal cytopathic effects. Supernatant fluids were then harvested and stored at –50°C until further passage. Cultures showing no cytopathic effects were followed for 14 days on primary chorion passage, and during later passages for 7-10 days. In subsequent passages 0.1 ml of the supernatant fluid from the previous chorion culture

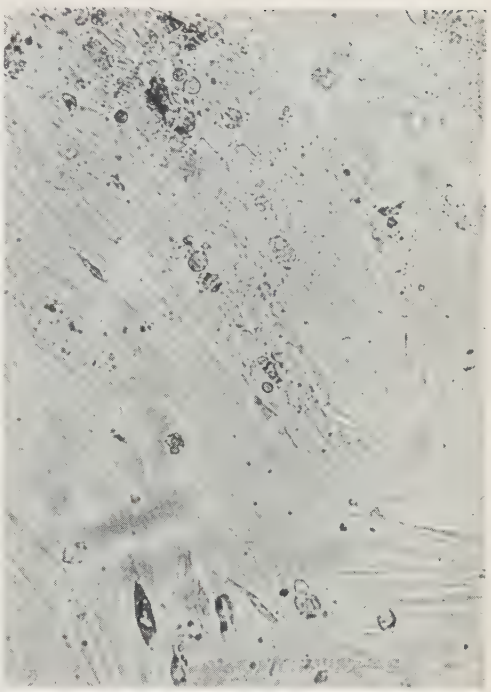

FIG. 1. Normal chorion culture (unstained) 5 days old showing an area composed largely of fibroblast-like cells (magnification × 150).

was transferred. All viruses, except salivary gland virus(5), tested were carried through a minimum of 5 tissue culture passages. Each 5th chorion passage was further inoculated into an appropriate susceptible host system, and those viruses producing cytopathic effects in chorion cells were found to produce pathologic changes in these different host systems compatible with each respective virus. Cultures inoculated with human salivary gland virus were incubated for as long as 17 days before first cytopathic effects were noted. When this change was marked, the adhering cells were scraped from glass with a rubber policeman, and passage was made with cells suspended in supernatant fluid. Salivary gland virus obtained from Dr. W. P. Rowe (5) was carried through 2 consecutive chorion passages, reisolated in human embryonic skin-muscle culture, and passaged once again in chorion. Pools of representative strains of those viruses that induced cytopathic changes in chorion were prepared by inoculating several chorion tube cultures with 0.1 ml fluid from the 5th, or in one case the 6th, chorion

TABLE I. Chorion Cell Susceptibility to Viruses.

Virus	Type	Virus source	Cytopathic effects: Epithelial cells	Cytopathic effects: Fibroblast cells
Poliovirus	1,2,3	Monkey kidney	+	+
Coxsackie B	1,2,3,4,5	*Idem*	+	±*
Coxsackie	A9	"	+	+
Adenovirus(7)	1,2,3,4,5,6,7,9†	HeLa or KB	+	±
Herpes simplex	—	Mouse brain	+	+
ECHO(6)	1,3,4,6,11	Monkey kidney	+	±
Salivary gland virus (5)	—	Human embryonic skin muscle	—	+
Influenza	A, B	Monkey kidney	—	—
"	C	Egg	—	—
Mumps	—	"	—	—
ECHO	2,7,8,9,10,12,13,14	Monkey kidney	—	—
Rabies	—	Mouse brain	—	—

* ± indicates cytopathic effect in fibroblast cells was not consistent on passage.
† On repeated trials adenovirus type 4 (RI-67) has not produced cytopathic changes beyond the second transfer.

passage. Titrations using these prepared 6th or 7th chorion passage pools as inocula were then performed with 4 tubes per 10-fold dilution. Chorion and at least one other susceptible tissue culture system were used.

Results. In the freshly prepared chorion cell suspensions 2 types of rounded cells were seen, a larger one about the size of a human polymorphonuclear leucocyte and a smaller cell approximately the size of a red blood corpuscle. After incubation of 4-6 days a sheet of mixed epithelial-like and fibroblast-like cells was formed (Fig. 1).

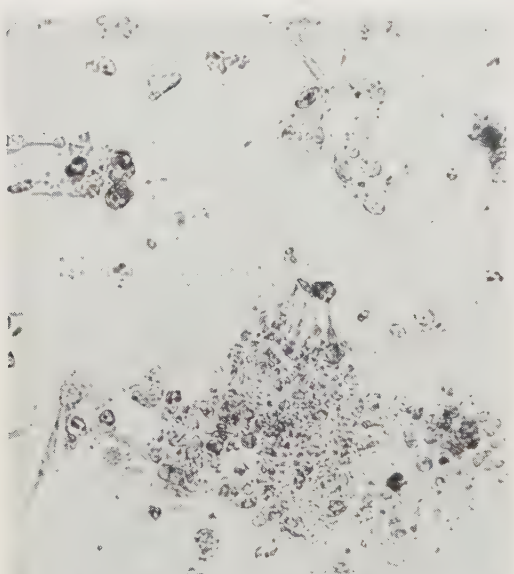

FIG. 2. Chorion cell culture (unstained) 60 hr after inoculation with poliovirus type 1 (magnification × 150).

The virus spectrum for the viruses tested is shown in Table I. Polioviruses types 1, 2, and 3 produced firstly cellular hypergranularity and rounding, and secondly, cell destruction in both epithelial and fibroblast cells (Fig. 2). The cytopathic effect of Coxsackie A9 could not be distinguished from that of the polioviruses. All 5 Coxsackie B and ECHO(6) viruses 1, 3, 4, 6, and 11 produced rounding of epithelial cells sparing many of the fibroblasts (Fig. 3). The adenoviruses (7) induced characteristic cellular clumping, and nuclear granularity seen also in HeLa and monkey kidney cell cultures, but the chorion fibroblasts were irregularly involved in adenovirus infection. Cell clumping and cytoplasmic stranding without marked nuclear hypergranularity were usually seen within 24 hrs. after inoculation with herpes simplex (Fig. 4). Salivary gland virus affected only the fibroblast cells of the culture which became enlarged, rounded, and opaque; first, only in an initial focus, but after 4-6 days, the entire cell sheet showed this effect. Influenza viruses A, B, and C, mumps virus, ECHO viruses 2, 7, 8, 9, 10, 12, 13, 14, and rabies virus produced no cytopathic changes, and subsequent inoculation into a known susceptible system revealed no virus.

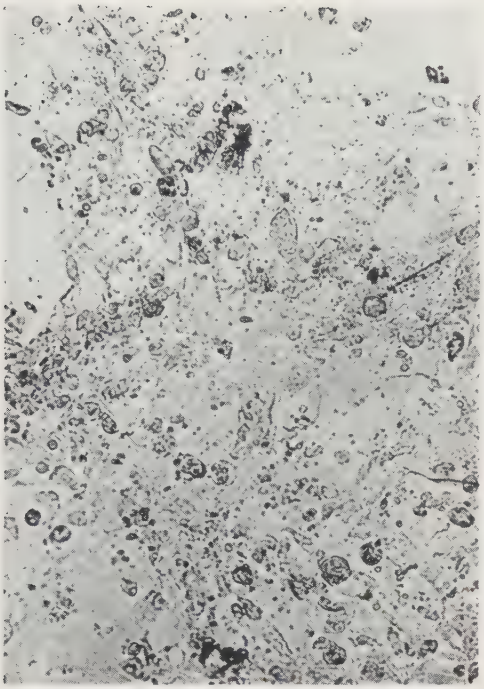

FIG. 3. Chorion cell culture (unstained) 5 days after inoculation with ECHO-6 virus (magnification × 150).

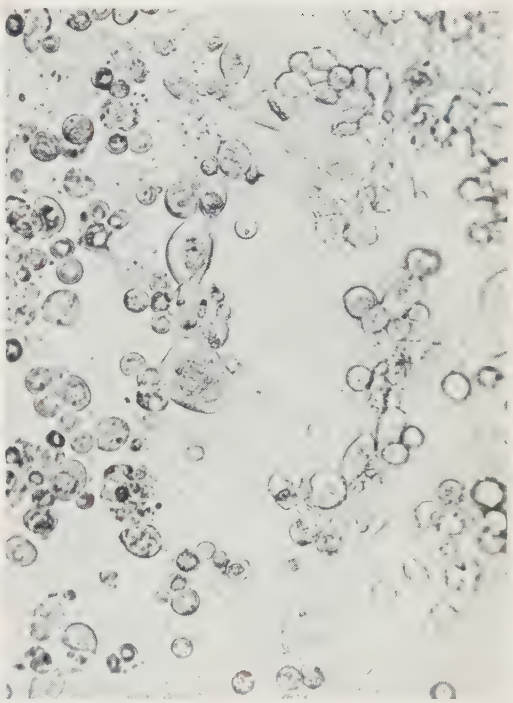

FIG. 4. Chorion cell culture (unstained) 72 hr after inoculation with herpes simplex virus (magnification × 150).

Limited work with vaccinia indicated that cytopathic effects in both epithelial and fibroblast cells are also regularly produced with this virus.

Table II shows the tissue culture infectious dose-50 ($TCID_{50}$) end-points with representative viral strains of known titer, namely poliovirus-1(10^6), Coxsackie B-3(10^5), Coxsackie A9($10^{5.5}$), herpes ($10^{2.6}$), adenovirus-3($10^{3.5}$), and ECHO-6(10^6) viruses. The results indicated that there had been propagation of these viruses in chorion cell cultures, and titers obtained in chorion were comparable to titers obtained in the other tissue culture systems tested with the same inoculum.

Discussion. Chorion cell cultures have been shown to be a ready and feasible source of mixed epithelial and fibroblast elements. The virus spectrum of epithelial cells was similar to that of the HeLa and human amnion cells. A single amnion usually yields approximately 250 tissue culture tubes, but by separate processing of the chorion from the same placenta, the number of available cultures can be increased by another 250. Moreover, the fibro-

TABLE II. Representative Titrations of Some of the Viruses Inducing Cytopathic Changes in Human Chorion Cells.*

Virus	Consecutive passages in chorion	Chorion	Amnion	Monkey kidney	HeLa
Poliovirus-1	6	4.3		4.8	
Coxsackie B3	6	4.5		6.0	4.3
A1	6	3.7		3.7	
Herpes	7	3.7	3.7		
Adenovirus-3	6	1.7		1.5	
ECHO-6	6	2.0		3.5	

* $TCID_{50}$/0.1 ml calculated by Reed-Muench method after observation period of 10 days.

blasts of the chorion offer the possibility of recognizing viruses which do not grow in epithelial cells, such as salivary gland virus. The fibroblasts have been maintained with infrequent medium changes for more than a month. The fact that chorion cultures contained 2 types of cell made recognition of cytopathogenic effect more difficult than with cultures of a single-cell type. After 2-3 weeks the epithelial cells were often shed leaving a relatively pure culture of fibroblasts.

Summary. 1. Chorion cell cultures of mixed epithelial and fibroblast-like cell types have been grown regularly in tissue culture. 2. Susceptibility of these cells to various viruses has been explored. Polioviruses types 1, 2, & 3; Coxsackie B1, B2, B3, B4, and B5; Coxsackie A9; adenoviruses 1, 2, 3, 4, 5, 6, 7, and 9; herpes simplex; ECHO viruses 1, 3, 4, 6, and 11; vaccinia; and human salivary gland virus have been found to induce cytopathic changes in chorion cell cultures.

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Some Normal Laboratory Values in the Dog. (23127)

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As part of preclinical evaluation of a potential drug in our laboratories the effect of long-term administration of the agent is observed in dogs. Because it is essential to know whether changes in chemistry or hematology are developing in response to a challenge with the therapeutic agent, control data are obtained on dogs prior to initiation of the agent. A compilation of these control data is presented herein. There is in the literature little systematic information concerning clinical chemical or hematologic norms in the dog in spite of extensive use of this species in laboratory work. Albritton(1) has made a critical selection of blood values from the literature, but many of the data were based on small populations, and each component was usually measured in a different group of dogs maintained under different conditions of diet and handling. This paper presents data obtained on blood and urine components in a large number of "normal" dogs under certain specified conditions. It is to be hoped that this may permit more useful correlations than would a mere compilation of the results of various tests that are at present scattered throughout the literature.

Methods. Female mongrel dogs were selected from stock on the basis of apparent good health, were dewormed, inoculated against distemper and maintained in metabolism cages for several weeks. A 500-g portion of food (25% horse meat in commercial dog food) was made available for one hour each day, after which remaining food was removed and weighed. Water was available at all times. After the dogs had become accustomed to handling and were of constant or increasing weight, weekly chemical and hematologic values were obtained prior to the daily meal. An extra 500 ml of water was given by gavage on the day preceding chemical analysis to insure an adequate volume of urine. Blood was drawn from the jugular

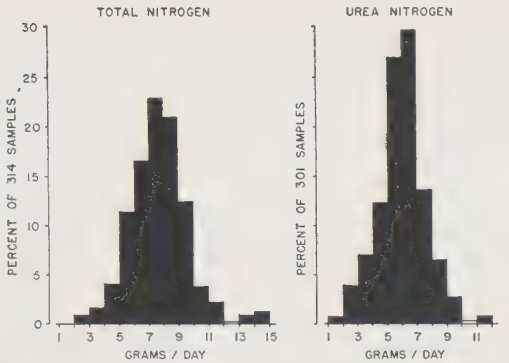

FIG. 1. Daily excretion of nitrogen by normal dogs.

vein; heparin was used as anticoagulant. When the results of 3 successive tests indicated reasonably stable values, the dog was considered to be ready for use in evaluation of a drug. The data presented below are the results of these 3 successive "triplicate control" values in over 100 dogs (total >300 determinations), except in the case of Table I. *Total and non-protein nitrogen* were determined by the method of Koch and McMeekin. *Protein* was calculated from the above as N x 6.24. *Urea* was converted to ammonia with urease and color developed with Nessler's reagent. *Creatinine* was determined with alkaline picrate. *Glucose* was determined on a tungstate filtrate with Benedict's reagent. *Total bilirubin* was measured by the method of Malloy and Evelyn, *cholesterol* by the procedure of Abell, *alkaline phosphatase* using β-glycerophosphate substrate prepared according to Shinowara, Jones and Reinhart, *phosphate* by the method of Fiske and Subba-Row, *potassium and sodium* using a Baird flame photometer with internal lithium standard, and *thymol turbidity* according to Maclagan. Literature references for these procedures may be found in Hawk, Oser and Summerson(2). Standard hematologic staining and counting technics were used. Hematocrit and sedimentation rates were measured using the Westergren method. Hemoglobin was determined by the method of Evelyn(3) adapted to the Coleman Junior Spectrophotometer.

Results. In a normal distribution, 67% of a population will fall within one standard deviation, and 95% will fall within 2 standard deviations. If a normal distribution be assumed for the type of data presented herein, one may approximate to the 95% range by excluding the top and bottom 2½% of observed values. This is the "95% range" as used below. It should be stressed that the data are derived from 3 tests on each individual, and that both chemical and hematologic values are obtained from the same dogs.

Chemical Results. Urine. Distribution of urinary excretion of nitrogen is shown graphically in Fig. 1 and 2. Total nitrogen excretion is shown for 314 twenty-four-hour collection periods on 105 dogs. The shape of the distribution appears to be nearly normal; 60% of the samples contain between 6 and 9 g. Since the diet provides 7.6 g of nitrogen/day, most dogs would seem to be in nitrogen balance. Urea nitrogen excretion is less variable than total nitrogen. Two-thirds of the samples are in the range of 5 to 7 g of urea nitrogen/day. The difference between total and urea nitrogen excretion for individual samples was, in most cases, between 1 and 2 g/day. Excretion of creatinine was more variable than that of either urea or total nitrogen (Fig. 2). When the creatinine excretion for individual samples was plotted against urea excretion, a positive correlation was apparent. On the other hand, no correlation was observed between urine volume and amount of creatinine excreted, although such a relation is often assumed to exist in the human.

The excreted volume ranged from less than 200 ml to more than 1200 ml. The urine pH

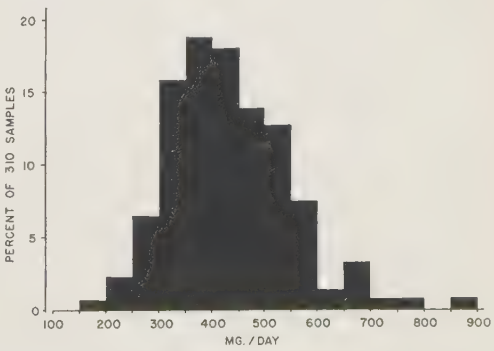

FIG. 2. Daily excretion of creatinine by normal dogs.

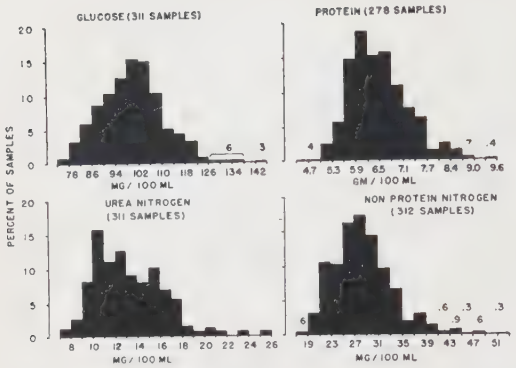

FIG. 3. Concentration in heparinized plasma of normal dogs.

was between 6.5 and 7.5 for 80% of the samples; the 95% range was 6.2 to 8.4. No positive bile tests were observed, and in only 5 samples was protein found by the acetic acid test. In 23 cases, positive Benedict's tests were seen. More than half of these were in one group of 12 dogs and are believed to represent an error of judgment in reporting as "positive" concentrations of less than 0.1 mg/100 ml.

Blood. Data for 4 common constituents of plasma are presented in Fig. 3. Sixty per cent of the glucose values were in the range of 90 to 110 mg/100 ml; 95% ranged from 80 to 120 mg/100 ml. The urea nitrogen distribution curve differed considerably from the statistically normal, but most values fell between 8 and 18 mg/100 ml. Two-thirds of the protein values fell between 5.6 and 6.8 g/100 ml. These limits are about one gram

TABLE I. Plasma Constituents Determined in a Limited Number of Normal Female Mongrels, Selected and Maintained as Described in Text. Except for cholesterol, 2 or 3 weekly samples were drawn on each animal.

Substance	No. of samples	Mean	"95% range"	
Bilirubin	69	.09	0 - 2	mg/100 ml
Cholesterol	20	193	136 -236	"
Creatinine	104	1.0	0.8- 1.2	"
Phosphorus, total	61	4.3	1.4- 6.5	"
Phosphatase, alkaline	61	4.2	1.7- 7.4	units
Potassium	24	4.1	3.9- 4.3	mEq/liter
Sodium	24	147	143 -150	"
Thymol turbidity	102	0.9	0 - 1.8	units

lower than have been reported elsewhere(1). The 95% range for non-protein nitrogen was 20 to 38 mg/100 ml. A plot of protein against non-protein nitrogen in individual samples showed no correlation between these components. The high values for glucose, urea, protein and non-protein nitrogen are scattered among the population and are not attributable to consistently high replicate values in a few animals.

A limited number of determinations of constituents listed in Table I has been made. The results may have lower validity than those of Fig. 1, 2 and 3, since a smaller number of dogs is represented and since all tests were not performed on a single group. Except for cholesterol, for which single values were obtained, the data of Table I include results of 2 or 3 weekly samples from each animal. In performing alkaline phosphatase determinations, it was found that the final pH of the buffered substrate-plasma mixture was actually between 9.6 and 9.8. Since it has been demonstrated(4) that the optimum pH is 9.5, these values are probably about 10% low.

Liver function was measured by the bromsulfalein test in 90 tests on 53 dogs. Retention at 30 minutes was less than 10% in all cases, and less than 3% in ¾ of the tests. Considerable data on kidney function in the dog have been presented in an earlier paper from this laboratory(5).

Hematologic tests. Fig. 4 summarizes results of hematology studies in 3 weekly control tests in over 100 dogs. The "95% range" is obtained by excluding the top and bottom 2½% of the values. Control data have been examined with respect to spread of the 3 weekly serial determinations on an individual dog. In general, the values for a particular dog did not vary greatly from week to week. Three hundred sixty *erythrocyte counts* were made on 120 dogs. The 95% range was 4 to 8 million/cu mm, but in only 11 dogs was the range of variation more than 1 million in the 3 weekly counts.

Hemoglobin varied more widely, the 95% range being 13 to 20 g/100 ml. In many dogs changes of 1 to 2 g were observed, but in a number of cases this was a consequence

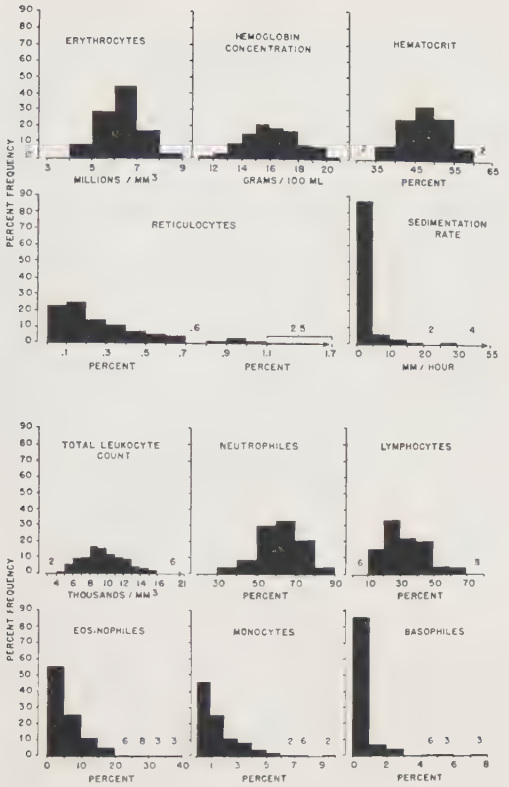

FIG. 4. Triplicate hematologic values in normal female mongrel dogs.

of increases in hemoglobin in dogs whose physical condition was obviously improving during the study period.

Hematocrit values are in accord with literature reports in this species. For any given dog, the 3 serial values were remarkably constant; in only 7 cases was the spread more than 10% among the 3 weekly determinations. The percentage of *reticulocytes* varied greatly, and a marked spread of values was often seen in individual dogs. The 95% range is meaningless in this instance; in one-half of the dogs, the count was less than 0.3% reticulocytes.

Sedimentation rates (Westergren) were zero in most cases. In 14 dogs, the sedimentation rate changed by 10 mm/hr from one week to another, but in one-half of these cases, the initial value was elevated and fell to normal in subsequent weeks.

The 95% range for *leukocytes* appeared to be from 5000 to 15,000/cu mm in this series. In general, the 3 determinations of total leukocyte count on an individual dog remained within 4000/cu mm, although in 3 dogs the count varied in a range of 8000/cu mm. A range of 9000 to 24,000/cu mm with a mean of 16,000 is reported in the literature(1). The lower values may reflect a lower incidence of intercurrent infection attributable to the selection and management of the dogs.

The percentage of *lymphocytes* and *neutrophils* ranged widely, with values as low as 50 neutrophils and as high as 50% lymphocytes in an appreciable portion of the population. For any particular dog, however, the percentage of either element changed only slightly from week to week; in only 10 cases was the range greater than 20%.

The *eosinophil* percentage was high in a few dogs, perhaps because of parasitism. Whether high or low, the count tended to remain constant at a value between zero and 10%. In only 8 dogs was the spread among triplicate values greater than 10%.

The range for *monocytes* was zero to 5%; within this range, a wider individual variation occurred from week to week than was anticipated. The 20 highest values were not restricted to a few dogs, although 4 dogs had more than 4% monocytes on 2 out of 3 counts.

A *basophil* count of zero was most frequent. The higher values represented sporadic high counts on a number of animals, rather than consistent elevation in a few.

Discussion. The present report defines the range of "normal" values to be expected within a large group of apparently healthy female dogs on a standard diet, using recognized procedures. The results are in general agreement with those compiled by Albritton(1) from a critical survey of the literature. A detailed comparison is beyond the scope of this report, involving questions of methodology, dietary states, and semantic difficulties associated with the word "normal."

An important feature of the ranges reported here for blood is their resemblance to those tabulated elsewhere(1,6) for the human. The "normal" distribution for most hematologic values is higher for the dog than for the human. Amounts of the chemically determined

constituents are similar to those of man. This would not necessarily be the case were other tests employed. Thus, in a number of samples in which thymol turbidity values were normal, the cephalin flocculation test was invariably positive, making the latter an invalid criterion for normal liver function in the dog. The differences may be a consequence of the dissimilar electrophoretic patterns that have been observed with canine and human serum.

The values observed for sodium and potassium in these "normal" dogs are higher than found in this laboratory for "trained" dogs that have been subjected to frequent venoclysis and venipuncture. It should be noted that whereas in the human the K^+/Na^+ ratio in erythrocytes is 220 times that in plasma, the K^+/Na^+ ratio in erythrocytes in dogs is similar to that in plasma. These considerations must be kept in mind in evaluating the importance of hemolysis in plasma samples and the significance of determinations of Na^+ and K^+ in whole blood.

The differences in renal clearance values between dog and man and the closely associated differences in metabolism of drugs are illustrated abundantly in the literature and need not be summarized here.

Summary. Hematologic and clinical chemical data were obtained on 3 occasions in over 100 apparently healthy female mongrel dogs. Procedures are described and the normal ranges presented.

The authors wish to express their appreciation to members of the Pathology and Pharmacological Chemistry Departments, to whom should go much credit for the chemical and biological phases of this report.

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Plasminogen in Pancreatic Disease and in Pancreatic Secretion. (23128)

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Plasmin is a proteolytic enzyme, normally existing in blood as inactive precursor, plasminogen(1). The latter may be transformed to plasmin by streptokinase(2) or other kinases(3-5). Plasminogen has been reported to be increased in a number of pathological and physiological conditions and, in some cases, blood has been found to possess spontaneous proteolytic activity, presumably due to presence of plasmin itself(1,6-14). In one report, excessive blood proteolytic activity was found in a patient undergoing surgery for carcinoma of the pancreas(15). Because several other serum enzymes are increased in pancreatic disease, it was thought of interest to investigate serum plasminogen levels when the pancreas was in an abnormal state. For the sake of clarity, several of the terms used are defined. *Plasmin.* Spontaneous proteolytic activity of blood; such activity is ascribed generally to plasmin. However, one should be mindful that characterization of this enzyme has not been sufficiently specific to rule out the possibility that this phenome-

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non may be caused by some other proteolytic enzyme. *Plasminogen.* Proteolysis brought about through activation by streptokinase; as determined here, it also includes any plasmin activity, if present.

Methods. Pancreatic and fat necrosis was produced in 12 dogs by one of 2 methods, and blood samples were drawn before and after operation for determination of serum plasmin, plasminogen and amylase. *Bile injection.* Bile from the dog's own gall bladder (5-10 ml), was aspirated and injected under pressure into the main pancreatic duct which then was ligated. Most of these animals died with hemorrhagic necrosis of the pancreas and abdominal fat necrosis. *Pancreatic duct section.* In aseptic operations, the distal end of the main pancreatic duct was excised together with a piece of duodenal wall including mucosa, and the defect in the duodenum was closed(16). The open duct permitted pancreatic secretion to drain freely into the peritoneal cavity, and the presence of duodenal mucosa on the sectioned duct made activation of trypsinogen possible. Most of these animals developed abdominal fat necrosis and died. The animals in both series received antibiotics post-operatively. *Determination of Plasminogen.* Hemoglobin substrate†: Trypsin substrate was prepared according to Orringer *et al.*(17), except that 300 g of urea was used instead of 400 g. Streptokinase (Varidase, Lederle‡): The amount in a vial was dissolved in 0.85% saline, so that 1 ml contained approximately 5000 units of streptokinase. Plasminogen in 2.5 ml of serum was precipitated with the euglobulin fraction at pH 5.2(18), and the precipitate dissolved in 2.5 ml of veronal buffer (pH 7.4)(19). Varidase (2.5 ml) was added to this solution and the tube placed in a water bath at 37°C for 10 minutes to obtain activation of the plasminogen into plasmin (20); 2 ml of the plasmin-Varidase preparation was then added to 5 ml of hemoglobin substrate and the whole incubated at 37°C for 2 hours. Inactivation of the enzyme with 0.3 N trichloracetic acid, filtration (Whatman #50 is recommended), preparation of a "control" or blank filtrate and development of color by phenol reagent are described by Anson under estimation of trypsin(21). Intensity of color was measured in Evelyn photoelectric colorimeter, (540 mμ filter). The results, read against a standard tyrosine curve, are expressed as mg of "tyrosine" released by amount of enzyme contained in 1 ml of serum. *Plasmin*: The method is the same as for plasminogen, except that veronal buffer was substituted for Varidase. *Serum amylase* was measured by the method of Somogyi, and results are expressed according to his definition of the amylase unit(22). Phosphate buffer (pH 7.2) was used to dilute the dogs' sera; the copper-reducing action of starch filtrates and glucose content of sera were determined iodometrically, using 2 ml of filtrate (23).

Results. As seen from Table I, a postoperative increase in both plasminogen and amylase was noted in all animals except one (No. 11), in which serum amylase failed to rise appreciably, because the bile had been injected into the peripancreatic tissues and only slight fat necrosis was present. In most dogs (exceptions No. 2, 6, 11), the percentage rise in amylase was greater than that of plasminogen, and in some (No. 3, 7, 10, 12), the latter assumed a downward trend, while the amylase remained high. In view of the seeming non-specificity of the various conditions in which increased blood proteolytic activity has been observed, it is possible that any increase noted here might be the result of tissue breakdown (necrosis) or of acute state of animal immediately following operation (shock, "stress") rather than to any effect specific for the pancreas only(6,7,24,25). Thus, this reaction may well be a secondary one.

In 7 animals, free serum plasmin was determined also; in 3 (No. 7, 11, 12), no free plasmin was present in the controls, but relatively high values appeared when pancreatic necrosis developed; in 2 (No. 9, 10), free plasmin was found before operation, followed by increases afterwards.

† Bovine Hemoglobin Enzyme Substrate Powder, Wilson Laboratories, Chicago, Ill.

‡ Varidase was furnished through the courtesy of Lederle Laboratories, Pearl River, N. Y.

TABLE I. Plasmin, Plasminogen, and Amylase in Dog's Serum before and after Operation to Produce Pancreatic or Fat Necrosis.

Dog No.	Blood sample time (hr)		Plasmin	Plasminogen	Amylase
1	Bile injection	0		.0153	2482
		12		.0595	9263
		24		.0510	14111
2	*Idem*	0		.0085	2021
		6		.0663	7569
3	"	0		.4471	3994
		2		.6290	30017
		24		.2822	31343
4	Pancreatic	0		.0986	3206
	duct section	21		.3392	15642
5	*Idem*	0		.3978	2797
		4		.5117	3932
		21		.4608	12076
6	Bile injection	0	.0	.1547	1048
		2	.0	.2737	2028
7	*Idem*	0	.0	.0527	1586
		2	.0918	.0493	5816
		8	.0	.1445	7954
		22	.0306	.1003	11180
8	"	0	.0	.0187	1418
		3.5	.0	.0349	3879
9	"	0	.0247	.4208	1604
		7.5	.1998	.8404	6087
10	"	0	.0136	.2244	2199
		7	.0391	.2669	18623
		19	.0	.2822	18983
		22	.0196	.2499	18623
11	"	0	.0	.2686	3240
		7	.0493	.3638	4030
12	"	0	.0	.0595	2041
		3	.0442	.2091	10204
		7	.1046	.1088	11216

All animals inj. with bile (except #11) showed pancreatic and fat necrosis.

All animals with severed pancreatic ducts showed fat necrosis.

More blood samples were taken, but only the most significant are presented.

Plasminogen was determined in the serum of 7 normal adults and of 4 patients with high serum amylase, but no patient exhibited increased plasminogen when compared with normal controls.

Obviously, the question arose as to whether plasminogen is present in the pancreatic secretion. Treatment of samples of pancreatic juice from 4 dogs with streptokinase (Varidase) brought about no increase in proteolysis over that caused by spontaneous proteolytic activity of the juice itself. On the other hand, when each sample of secretion was treated with enterokinase,§ greatly increased proteolysis, over and above that shown spontaneously, resulted. These results indicate that the enzyme activated by streptokinase and that activated by enterokinase are not the same. This conclusion concurs with a previous report by Kaplan(26) in which somewhat different methods were used.

Summary. 1. Serum plasminogen and amylase levels were measured in 12 dogs in whom pancreatic necrosis and/or fat necrosis was produced. An increase of plasminogen along with the expected increase in amylase was noted under the conditions described. 2. Samples of pure pancreatic juice obtained from 4 dogs were tested for the presence of plasminogen. No proteolytic activity due to the activating effect of streptokinase was present by the method employed.

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Strain Differences in Response of Mice to Mammary Gland Stimulating Hormones.* (23129)

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Most species of mammals require a combination of progesterone and estrogen to obtain full development of the mammary gland while in a few species, notably the guinea pig and the monkey, estrogen alone will cause complete development(1). Possible strain differences in mammary gland growth responses within a species are less well known. In studies on mammary cancer susceptibility in mice, strain differences have been found to exist. Also Richardson(2,3) and Richardson and Cloudman(4) have reported variation in extent of rudimentary duct development in various inbred strains of male mice. Mixner and Turner(5) have used ovariectomized virgin albino mice rather extensively in experiments on factors responsible for and influencing mammary lobule-alveolar growth. In these studies a single strain of albino mice had been used. In subsequent studies a second strain of mice showed a marked difference in mammary growth responsiveness as compared to the first strain of mice. Other strains of mice were then tested in this respect.

The present paper deals with strain differences in the mammary lobule-alveolar growth responsiveness of mice to estrone in conjunction with both progesterone and pituitary mammogenic lobule-alveolar growth factor.

Procedure. Two anterior pituitary extracts and progesterone were assayed for their ability to promote mammary lobule-alveolar growth in 4 strains of mice. Our original strain of albino mice (designated "Schwing") were obtained from Edward Schwing, Harrison, Ohio, who stated that his strain of mice was started about 1906 from a pair of mice. A second strain of mice (designated "Swiss") was obtained from Rockland Farms, New City, N. Y. The third strain of mice (designated "Kansas") was obtained from Mrs. Ray Whitehouse of Hardtner, Kansas, who stated that the original pen of mice was obtained from James W. Howck & Co., Tiffin, Ohio. The last strain of mice (designated "Sutter") was obtained from Arthur Sutter, Springfield, Mo., who advertised them as "Rockland All-Purpose Strain White Mice." The criterion of response in the assay method used was the per cent of ovariectomized mice showing a minimal mammary lobule-alveolar growth response after 10 daily injections of standard anterior pituitary preparation or progesterone, each injected in conjunction with a daily dose of 0.75 μg estrone. A mouse unit of mammogenic material is that amount of material required per mouse which will cause 50 $\pm$ 10% of 10 or more mice to show this minimal stimulation of the mammary glands.

Results. Mice of the 4 strains selected

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TABLE I. Mammary Gland Growth Responses of Various Strains of Mice.

Preparation and total dosage* (mg)		Mice: Strain	Mice: No. of animals	Mammary lobule-alveolar responses: +	Mammary lobule-alveolar responses: —	Mammary lobule-alveolar responses: % positive
Lot 13, initial ext. of ant. pituitary, stockyard cattle	7.5	Schwing	35	18	17	51.4
	"	Swiss	15	8	7	53.3
	"	Kansas	11	3	8	27.3
	"	Sutter	11	3	8	27.3
	15	"	13	7	6	53.8
Lot 15, acetone-ether, dried ant. pituitary, pregnant cattle	15	Schwing	27	14	13	51.9
	"	Kansas	12	1	11	8.3‡
	"	Sutter	34	9	25	26.5‡
Progesterone	1	Schwing	21	10	11	47.6
	1	Swiss	15	4	11	26.7
	1	Kansas	12	1	11	8.3‡
	1	Sutter	24	6	18	25.0†
	2	"	12	6	6	50.0

* Plus 0.75 μg estrone/day.
† Response is significantly different from the Schwing strain at the 5% level.
‡ —*Idem*— 1% " .

were each injected with a total of 7.5 mg of Lot 13, initial extract of anterior pituitary, stockyard run of cattle, which amount was a mouse unit according to assays using the Schwing strain. In addition a second group of Sutter mice received 15.0 mg or 2 mouse units of this same extract per mouse (Table I). The Schwing and Swiss mice gave assay responses of 51.4% and 53.3% respectively while the Kansas and Sutter mice each gave 27.3% positive responses indicating that their magnitude of response was about one-half that of the first two strains. By doubling the dose (15 mg) for the Sutter mice, unit assay response (53.8%) was obtained.

Mice of the Schwing, Kansas and Sutter strains were each injected with a total of 15 mg of a second anterior pituitary preparation designated Lot 15, acetone-ether dried anterior pituitary, pregnant cattle, which amount was a mouse unit according to the Schwing mice assay (Table I). The Kansas and Sutter mice gave responses of 8.3% and 26.5% respectively, indicating again that their magnitude of response was one-half (or less) that of the Schwing strain of mice.

Mice of all strains were injected with a total of 1 mg of progesterone, this being a mouse unit according to the assay on Schwing mice. A second group of Sutter mice received 2 mg or 2 mouse units of progesterone (Table I). The Sutter, Swiss, and Kansas mice gave 47.6, 26.7, 8.3, and 25.0% mammary responses respectively with 1 mg of progesterone. The Kansas and Sutter mice responded in a similar manner to that of the previous experiments, the Swiss mice being inconsistent in this respect. The difference in mode of action of progesterone and anterior pituitary factor could readily account for the differing response of the Swiss mice to these preparations. It is interesting to note that the Sutter mice gave a control assay response (50%) with 2 mg (2 mouse units) as they did similarly with 15 mg (2 mouse units) of the first pituitary preparation.

A chi-square statistical analysis was used to determine whether the individual and collective responses of each of the Swiss, Kansas, and Sutter strains of mice differed significantly from that of the control Schwing strain (Table I). From this analysis it may be concluded that the mammary growth responses of the Kansas and Sutter strains differed in a highly significant manner ($P<0.01$) from the Schwing strain responses. The difference in the way the Swiss strain responded to anterior pituitary extract and progesterone, makes a comparison of the Swiss strain to the Schwing strain inconclusive.

Discussion. The cause or causes of these strain differences in mice in mammary gland growth responses are not known. It is possible that in some strains of mice the mam-

mary gland cells are more refractory to their direct growth stimulus than in other strains. This might be the case with the strains of mice which were deficient in their response to anterior pituitary materials. Since progesterone exerts its mammary growth effect through the anterior pituitary, it is possible that in some strains the pituitary is not optimally stimulated by progesterone, poorer mammary growth resulting. Further studies will have to be made to determine the possible roles of other endocrine glands such as the thyroid in the observed deficiencies in mammary growth.

Summary. Four strains of albino were studied as to mammary lobule-alveolar growth responses to anterior pituitary preparations and progesterone. Statistically significant differences in strain responses were found. The Schwing and Swiss strains of mice were definitely superior to the Kansas and Sutter strains in their response to anterior pituitary preparations. The Swiss, Kansas, and Sutter strains of mice were all inferior to the Schwing strain in response to progesterone.

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Effect of Some Pentoses on Growth of Lactobacilli.* (23130)

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Two recent reports(1,2) have emphasized the effect of certain pentoses in stimulating early growth of lactobacilli. In the report of Camien and Dunn(1) 3 pentoses were used individually. Often L-arabinose was somewhat more effective for *L. gayonii* and related species than was D-xylose or D-ribose, though the stimulatory effect of the individual pentoses differed somewhat with different cultures.

The present report deals with the growth response to a mixture of 3 pentoses shown by a collection of lactobacilli. The investigation was prompted by preliminary observations on a few cultures which indicated that homofermentative lactobacilli responded to pentoses quite differently than heterofermentative lactobacilli, particularly in a semisynthetic medium. It was found that this difference between the 2 fermentative types holds true, for the most part, for a larger number of lactobacilli and the degree of difference varies with the test medium.

Materials and methods. Two culture media were used for the tests. One was a semisynthetic medium containing 0.5% acid digest of casein plus added tryptophane, cysteine, serine, threonine, purines, pyrimidines, vitamins, acetate, inorganic salts, and 1.0% glucose which was autoclaved in the medium. The detailed composition was given previously(3) but in the present study a few changes were made: the hydroxyproline of the previous work was omitted and xanthine was used in place of hypoxanthine. For testing the effect of pentoses, a mixture of L-arabinose, D-ribose, and D-xylose was sterilized by heat, but apart from the medium. This solution was added to tubes of previously autoclaved medium in an amount sufficient to give 0.5% of each pentose. In performing the tests, one series of tubes contained only the 1% glucose, tubes of the other series contained 1% glucose plus 0.5% of each of the 3 separately-sterilized pentoses. Certain departures from the foregoing pro-

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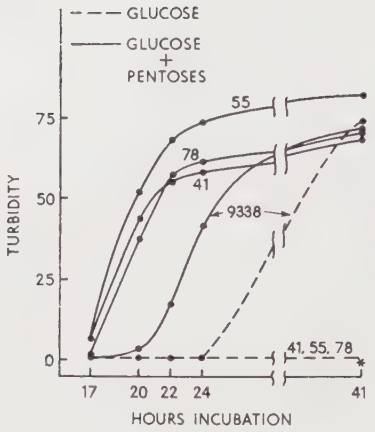

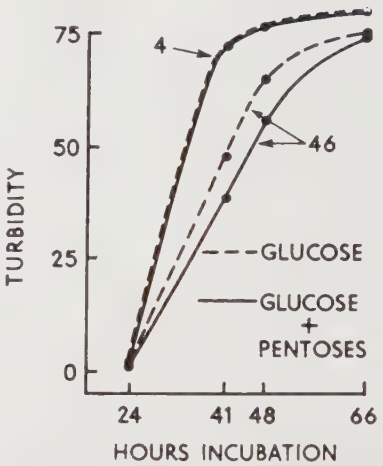

FIG. 1 (left). Growth response of 4 heterofermentative lactobacilli (*L. fermenti* strains 41, 55, 78, and ATCC 9338) in semisynthetic medium in presence of either glucose or glucose plus the 3 pentoses. Turbidity scale is based upon light transmission with a 580 mμ filter: 0 = clear tube, 65 to 80 = near maximal or maximal growth.

* Growth of cultures 41, 55, and 78 in glucose medium appeared in 41 to 48 hr and attained turbidity values of 65 to 73.

FIG. 2 (right). Growth response of 2 homofermentative lactobacilli (*L. casei* 46 and lactose-negative *L. casei* 4) in semisynthetic medium with either glucose or glucose plus pentoses.

cedure were made in some instances. In a few cases, 0.5% glucose was autoclaved in the medium while another 0.5% glucose was sterilized separately and added later. Also, in some later tests, the % of glucose in the one series was increased to equal total % of glucose plus pentoses. This and certain other departures from the usual procedure will be referred to later in connection with results. The second test medium was APT medium (Case) which is a modification of that of Evans and Niven(4). It is an enzyme digest of protein plus yeast extract, phosphate and other salts, polyoxyethylene sorbitan monooleate (Tween 80), and 1% glucose. For tests of the effect of pentoses, a separately sterilized mixture of L-arabinose, D-ribose, and D-xylose was added, as before, to previously autoclaved tubes of medium to give 0.5% of each pentose, in addition to the 1% glucose sterilized with the medium.

Thirty-seven lactobacilli were used. The homofermentative subgroup was represented by 12 strains of *L. casei*, 4 "lactose negative" *L casei*, 2 *L. casei*-like, 5 *L. plantarum*, and 3 *L. acidophilus;* in the heterofermentative subgroup were 9 *L. fermenti* and 2 from greenish discolored meat products.† Of the 37 cultures, 5 homofermentative and one heterofermentative were from the American Type Culture Collection. The others, aside from the 2 meat product cultures, had been obtained either from saliva or carious lesions and maintained for some years by monthly transfers in tomato juice yeast extract medium. Cells used for the tests were grown usually in the tomato yeast extract medium or, in some cases, in APT medium. For inoculation, either 0.001 or 0.0002 ml, secured by dilution of approximately 24-hour cultures, was used in most cases; in a few tests 0.01 ml was used. In each test, all tubes received the same amount of inoculum. Incubation was at 36°C, though 30°C was employed in a few instances.

Results. Growth of some representative lactobacilli in the semi-synthetic medium, with and without the 3 pentoses, is shown in the figures. The 4 heterofermentative lactobacilli in Fig. 1 ordinarily grew rather slowly in the semisynthetic medium, turbidity ap-

† For these 2 cultures the writers are indebted to Dr. J. B. Evans and Mr. R. H. Deibel of American Meat Institute Foundation.

TABLE I. Effect of Pentoses on Early Growth Response.

Species and No. of strains tested		Faster with pentoses		Essentially no diff.		Slower with pentoses	
		SS*	APT*	SS	APT	SS	APT
Homofermentative							
L. casei	18†	0	0	11	4	7	14
L. plantarum	5‡	0	0	0	0	5	5
L. acidophilus	3	0	0	3	3	0	0
Heterofermentative							
L. fermenti	9	9	9	0	0	0	0
From discolored meat products	2	0	0	2§	2§	0	0

* Letters refer to semisynthetic (SS) and APT medium.
† The 18 *casei* strains include 4 lactose negative *casei* and 2 *casei*-like cultures.
‡ *L. arabinosus* 17-5 (*i.e.*, *L. plantarum* ATCC 8014) is one of the 5 strains.
§ Tests were made at both 30° and 36°C; one of these cultures failed to grow at 36°C. On repeated tests of these 2 cultures there were a few instances in which growth was a little slower in the APT medium in the presence of pentoses. Since the difference was slight and was not observed at other times, these 2 cultures are classed in the "no difference" category under the APT medium.

pearing only after 24 to 40 hours or more, although heavy growth was attained later. These organisms in repeated tests in the semisynthetic medium responded more readily when the 3 pentoses were supplied along with glucose. Similar results were secured with 5 other strains classed as *L. fermenti*.

Results of tests with several species or varieties of homofermentative lactobacilli were different. In some cases growth was somewhat slower when the 3 pentoses were included in the medium; in others, the rate of growth was approximately the same. Results shown in Fig. 2 are typical of other *L. casei* cultures as well as of *L. plantarum* and *L. acidophilus*. In no instance did the 3 pentoses stimulate growth of any of the homofermentative lactobacilli.

In APT medium, which contains yeast extract, all of the lactobacilli produced speedier growth than in the semisynthetic medium; nevertheless, the effect of the pentoses was still evident and the results paralleled those obtained with the semisynthetic medium. Included in these tests were different-sized inoculations from cultures of varying ages from 16 to 48 hours.

A summary of results with all cultures in both experimental media is given in Table I. Growth of the homofermentative lactobacilli either was not appreciably affected by the pentoses or it was slower in their presence. Results with individual strains of *L. casei* differed somewhat in this respect in the 2 media. In no case, however, was growth of *L. casei*, *L. plantarum*, or *L. acidophilus* accelerated in the presence of the pentoses.

Of the heterofermentative lactobacilli, all 9 *L. fermenti* (ATCC 9338 and 8 strains of oral origin) exhibited pentose stimulation. The 2 cultures from discolored meat products, however, were not so affected. It has been pointed out by others(4,5) that these lactobacilli differ in a number of respects from the familiar heterofermentative lactobacilli encountered commonly in lactic fermentation of milk or in the mouth. Their lack of response to the pentoses also seems to set them apart from *L. fermenti*.

Results of some additional tests involving departures from the usual procedure can be summarized briefly. At 30°C, instead of 36°C, growth was slower but pentose stimulation of *L. fermenti* was evident. The pentose effect showed no apparent correlation with pentose fermentation as determined by the usual fermentation tests. The majority of the *L. fermenti* cultures did not ferment xylose or arabinose but most of them fermented ribose. All of them showed growth stimulation by pentoses irrespective of the pentoses fermented. Of the homofermentative cultures, *L. casei* and varieties did not ferment arabinose or xylose but fermented ribose; *L. plantarum* fermented arabinose and ribose, or arabinose, xylose and ribose.

Yet these homofermentative lactobacilli which fermented readily at least 2 or, in some cases, all 3 pentoses showed no growth stimulation in the presence of the pentoses and some of them were inhibited.

An increase in the amount of glucose in the medium to equal the percent of glucose plus pentose did not alter the result: *L. fermenti* cultures in increased glucose were not stimulated as were the same cultures when supplied the pentoses and a smaller amount of glucose. The stimulation evidently is not simply a matter of an increased amount of fermentable sugar. When glucose was omitted from the medium, leaving the 3 pentoses, most of the lactobacilli failed to grow while a few grew very slowly. Autoclaving of the 3 pentoses along with glucose in the semisynthetic medium, instead of separate sterilization and later addition of the pentoses, did not decrease their stimulatory effect for *L. fermenti;* on the contrary, it seemed to augment it in some cases. No detailed study was made of the effect of individual pentoses on *L. fermenti.* A few tests showed that the growth acceleration produced by single pentoses differed somewhat from one strain to another, somewhat in the manner noted by Camien and Dunn(1).

Summary. Growth of the heterofermentative *Lactobacillus fermenti* (9 strains) was stimulated by a mixture of 3 pentoses: L-arabinose, D-xylose, and D-ribose when added to glucose-containing media. The pentose stimulation was observed in two different media but was more evident in a semisynthetic medium than in a medium containing yeast extract (APT medium). In contrast, no such stimulation was observed with any homofermentative lactobacilli classed as *L. casei, L. plantarum,* and *L. acidophilus.* Some of these cultures were slightly inhibited by the pentoses. Two heterofermentative lactobacilli from discolored meat products differed from *L. fermenti;* their growth was not appreciably affected by the pentoses.

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Distribution of Radioactivity in Human Maternal and Fetal Tissues Following Administration of C^{14}-4-Progesterone.* (23131)

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During human pregnancy, the plasma concentration of progesterone is very low and does not parallel the rapid increase of its urinary metabolites(1). This observation raised some doubt as to whether pregnanediol and pregnanolone excreted into the urine during pregnancy are actually the metabolic products of progesterone. On the other hand, it could be explained by the assumption that progesterone disappears quite rapidly from blood circulation in pregnancy. The latter explanation seemed to be more likely since Klein and Ober(2) were not able to increase plasma concentration of progesterone (as determined by the Hooker-Forbes test) in plasma of pregnant women by intramuscular administration of very large doses of progesterone (1000 to 1200 mg), although urinary pregnanediol is excreted in relatively large amounts following such an injection.

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Our own studies(3) revealed that after intramuscular administration of C^{14}-4-progesterone during 11th and 17th week of pregnancy plasma concentration of radioactivity rose sharply to a peak value within 24 hours and declined more slowly thereafter over 9 to 12 days. The peak concentration of radioactivity in plasma was relatively low, whereas large amounts of radioactivity appeared in urine at the same time. The concentration curve in plasma closely resembled that found for urinary samples obtained at frequent intervals after injection of the tagged hormone. In view of this parallelism and the observation of Klein and Ober it appeared proper to assume that the rise in concentration of radioactivity in plasma was due to an increase in concentration of metabolites rather than to the hormone. Substantial amounts of radioactivity derived from injected C^{14}-4-progesterone are eliminated by the feces(3) after having been secreted into the gastrointestinal tract by way of the bile. However, prompt excretion of inactivation products of progesterone into urine and feces does not fully explain the fact that large intramuscular doses of progesterone do not raise plasma level of the hormone during pregnancy. Intravenous administration of progesterone is followed by a rapid decline of plasma concentration within 2 hours(4). No progesterone could be detected in the plasma 24 hours after intravenous injection(5) although pregnanediol is excreted over a period of several days(4,6). There is no fundamental difference in excretion patterns of pregnanediol in the urine after intramuscular or intravenous injections or progesterone, indicating that absorption of the hormone from an oily intramuscular depot must be a fast and efficient process.‡

The following experiments were undertaken to study the possibility that progesterone absorbed from an oily depot in the muscle diffuses rapidly into the tissues. In the experiments reported here, the distribution of radioactivity in maternal and fetal tissues removed at various time intervals following intramuscular injection of C^{14}-4-progesterone, was determined.

Methods. C^{14}-4-progesterone prepared by the method of Thompson *et al.*(8), and purified by chromatography on alumina, was administered intramuscularly to 3 pregnant patients. Therapeutic abortions were performed at various time intervals after the injections. Maternal and fetal tissues obtained at surgery were combusted to carbon dioxide in a vacuum combustion line and counted in an ionization chamber, using a vibrating reed electrometer according to the method of Brownell and Lockhart(9). All measurements were done for periods long enough to give a standard error of less than $\pm$ 5%.

Results. Patient C.C. This patient (gravida III, para II) was 37 years old and had a severe bronchial asthma which required almost constant medical care. Seven months before she became pregnant she had hyperthyroidism. Treatment with I^{131} and propylthiouracil resulted in a good therapeutic response, but it did not have a favorable effect upon the asthmatic condition. The patient was 11 weeks pregnant when 28.1 μc of C^{14}-4-progesterone were injected intramuscularly 48 hours before a therapeutic abortion was performed. The distribution of radioactivity found in maternal and fetal tissue is reported in Table I. The results are ex-

TABLE I. Radioactivity in Maternal and Fetal Tissue.

Dose of C^{14}-4-progesterone, 28 μc (intramusc.). 48 hr interval between inj. and therapeutic abortion. 11 wk of pregnancy.

Tissues	Wt, g	μc/g $\times 10^{-6}$	μc/ organ $\times 10^{-6}$	% of administered radioactivity in organ
Maternal				
Corpus luteum	3.67	153	562	.00200
Rest of ovary	2.58	45	116	.00041
Decidua		170		
Myometrium		368		
Fat		845		19.6 *
Skin		72		
Fetal				
Placenta	46.	52	2392	.00854
Liver	.62	26	16	.00005
Lungs	.25	10	3	.00001
Heart	.12	14	2	.000007
Intestine	.46	11	5	.00002
Brain	1.85	0		—

* Calculated from total body wt of patient.

‡ This is likewise true for estrogens since there is no difference between daily amounts of radioactivity excreted into urine after intramuscular and intravenous injection of C^{14}-labelled estrogens(7).

TABLE II. Radioactivity in Maternal and Fetal Tissue.

Dose of C^{14}-4-progesterone, 12 μc (intramusc.). 24 hr interval between inj. and therapeutic abortion. 17 wk of pregnancy.

Tissues	Wt, g	μc/g × 10^{-6}	μc/ organ × 10^{-6}	% of administered radioactivity in organ
Maternal				
Corpus luteum	2.45	71	175	.00146
Rest of ovary	4.60	18	83	.00069
Decidua		62		
Myometrium	531 *	41	21771	.18142
Fat		368		33.7 †
Skin		85		
Fetal				
Placenta	120	74	8880	.07400
Liver	8.25	0	—	
Adrenals	.70	61	43	.00036
Testes	.20	0		
Lungs	3.70	35	128	.00107
Heart	.65	18	12	.00010
Brain	12.00	0		

* Total wt of emptied uterus.
† Calculated from total body wt of patient.

pressed in μc/g of tissue. Table I also gives percentage of administered dose of radioactivity found in various organs. The myometrium, decidua and corpus luteum had relatively high concentrations of radioactivity whereas moderate amounts were detected in the rest of the ovary, maternal skin and fetal placenta. Relatively small amounts of radioactivity derived from labelled progesterone were found in all fetal tissues with the exception of the brain where no radioactivity was detected by the method used. However, by far the highest concentration of radioactivity was present in a sample of maternal fat obtained from subcutaneous tissue of abdominal wall. Assuming that 18% of total body weight of this patient consisted of fat, approximately 19.6% of the administered dose of radioactivity was present in the fat compartment 48 hours after administration of the tagged hormone provided that radioactivity was evenly distributed in the fat.

Patient J.O. This patient (gravida VIII, para V) was 37 years old and had a huge goiter for many years which enlarged quite rapidly during a 5-month period preceding date of admission to hospital. At that time she was 11 weeks pregnant. There were no symptoms of thyrotoxicosis. Growth of the goiter continued during the following weeks and the pregnancy was terminated during the 17th week because of possible carcinoma of the thyroid. The patient received 12 μc of C^{14}-4-progesterone intramuscularly 24 hours before surgery. The values of radioactivity found in the various tissues are shown in Table II. Again, the highest concentration of radioactivity was detected in a sample of maternal fat. Only moderate amounts of radioactivity were present in the myometrium, decidua, corpus luteum, maternal skin and fetal placenta. Concentration of radioactivity in fetal organs was lower than that found in maternal tissues. No radioactivity could be detected in the fetal liver, testes and brain. A relatively high concentration of activity was present in fetal adrenals when compared with that found in other fetal organs.

Patient M.P. This patient (gravida II, para 0) was 27 years old and had lupus erythematosus. The patient's condition became progressively worse during the first half of pregnancy and a therapeutic abortion was performed during the 18th week of gestation. This patient died of her diseases 6 weeks after pregnancy termination. She received 15.5 μc of C^{14}-4-progesterone intramuscularly 12 hours before the hysterotomy was performed. As shown in Table III, considerable amounts

TABLE III. Radioactivity in Maternal and Fetal Tissue.

Dose of C^{14}-4-progesterone, 15.5 μc (intramusc.). 12 hr interval between inj. and therapeutic abortion. 18 wk of pregnancy.

Tissues	Wt, g	μc/g × 10^{-6}	μc/ organ × 10^{-6}	% of administered radioactivity in organ
Maternal				
Corpus luteum	1.10	55	61	.00039
Decidua		46		
Myometrium	490 *	89	43610	.28135
Fat		228		17.7 †
Skin		168		
Fetal				
Placenta	111	115	12765	.08235
Liver	5.39	41	221	.00143
Adrenals	0.54	112	61	.00039
Lungs	5.51	14	77	.00050
Heart	1.02	7	7	.00004
Brain	18.4	0		—

* Wt of emptied uterus.
† Calculated from total body wt of patient.

of radioactivity were found in maternal fat and skin obtained from the abdominal wall during the operation. Comparatively small amounts of activity were present in the myometrium, decidua, and corpus luteum. The largest amounts of radioactivity in the fetal organs were found in the placenta and adrenals, whereas no activity could be detected in the fetal brain.

Discussion. Following intramuscular administration of C^{14}-4-progesterone to 3 pregnant patients the highest concentration of radioactivity was found in maternal fatty tissue. Assuming an even distribution of radioactivity in the fat of the body, about 17.7%, 33.7%, and 19.6% of the administered dose were present 12, 24, and 48 hours, respectively after administration of the labelled hormone. These calculations are based on the assumption that 18% of total body weight of the patients consisted of fat.

These findings indicate that progesterone and/or its metabolites diffuse promptly from the blood circulation into the fat of the body when this steroid hormone is administered intramuscularly. We have not determined whether the radioactivity found in fat was derived from the hormone or from its metabolites. However, it appears very likely that the radioactivity is principally due to the presence of unchanged hormone since Kaufmann and Zander(10) found relatively high concentrations of progesterone in fatty tissues of pregnant women which were 6 times greater than that found in fat of non-pregnant women during the luteal phase of the menstrual cycle.

Our results explain the following observations: (1) failure of large doses of progesterone injected intramuscularly to raise blood level of progesterone and rapid disappearance of progesterone from blood after intravenous injection; (2) delayed excretion of progesterone metabolites after intravenous injection; (3) the previous observation(3,11) that specific activity of urinary C^{14}-pregnanediol derived from injected C^{14}-acetate declined during a 48 to 72 hour period after removal of principal sources of the hormone, the placenta and the corpus luteum. We explained this phenomenon previously as a dilution of the radioactive pregnanediol with inert steroid molecules derived from the maternal adrenals. In view of our findings it is more likely that the urinary C^{14}-pregnanediol was diluted by molecules derived from progesterone stored in maternal fat.

It further demonstrates that erroneous conclusions may be drawn from the values obtained for urinary pregnanediol in regard to progesterone production. Pregnanediol may continue to be excreted into the urine in appreciable amounts although production of the hormone has already ceased, a fact which has been recently demonstrated in one of our patients who was in spontaneous labor(3,11).

It is of interest to note that only moderate amounts of radioactivity derived from tagged progesterone were found in the myometrium and the decidua in 2 patients with advanced pregnancy (17th and 18th week of gestation), whereas a relatively higher concentration was found for both tissues in a patient who was 11 weeks pregnant. The decidua as well as the myometrium are considered to be the most important target organs for progestational activity. In mice and rats a low level of radioactivity derived from C^{14}-21-progesterone was found in the uterus(12). Progesterone could not be isolated from the myometrium and the decidua of pregnant women when a very sensitive method of chemical assay was used(13). At present, we have no knowledge about the actual mechanism by which a hormone exerts its biological effect on the target organ.

Low to moderate concentrations of radioactivity derived from C^{14}-4-progesterone were found in the corpus luteum and the placenta. This is logical for these endocrine glands are unfavorable sites for absorption and storage of the hormone which they elaborate.

Radioactivity was found in all fetal tissues in relatively small amounts with exception of the brain (negative radio-assay in all 3 cases), the testes (negative assay in one case) and the liver (negative assay in one of 2 cases). The comparatively highest concentration of radioactivity in fetal organs was found in the adrenal glands of 2 fetuses 17 and 18 weeks old. Progesterone is regarded as an important intermediate of adrenal cortical steroids. However, only small amounts of corticoids

can be detected in fetal adrenal glands at this stage of embryonic life. On the other hand, the relatively high concentration of progesterone may be due to accumulation of lipids in the fetal cortex. However, histochemical studies indicate that lipoid material is present in only relatively small amounts in the fetal cortex, but these amounts may be still higher than that found in other fetal organs. In view of this finding it is of interest that Riegel *et al.*(12) found a relatively high concentration of radioactivity in the adrenals of mice after administration of C^{14}-21-progesterone.

Summary. C^{14}-4-progesterone was administered intramuscularly to pregnant women who were scheduled for therapeutic abortion. Distribution of radioactivity in maternal and fetal tissue removed at various time intervals following injections was determined. The highest concentration of radioactivity was found in the maternal fat. A rough calculation revealed that it contained about 17.7% of the administered radioactivity 12 hours, 33.7% 24 hours, and 19.6% 48 hours after administration of the labelled hormone. These findings indicate that progesterone and/or its metabolites diffuse promptly from the blood circulation into the fat compartment of the body. Only moderate amounts of radioactivity were found in the myometrium and the decidua, the 2 principal target organs of progesterone. Low to moderate concentrations of radioactivity were detected in the organs which serve as principal sources of the hormone, the corpus luteum and the placenta. Radioactivity was present in all fetal tissues in relatively small amounts with the exception of the fetal brain, and the testes.

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Assay and Some Properties of Kidney Transamidinase.* (23132)

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Transamidination in mammalian kidney slices and homogenates was first reported by Borsook and Dubnoff(1). The reaction has since been confirmed by other investigators with the use of isotopic technics(2-5).

This paper reports a simple assay for tissue transamidinase activity. The assay was used to determine the following effects: addition of sulfhydryl inhibitors both *in vivo* and *in vitro,* substitution of canavanine for arginine as one of the substrates, *in vitro,* and substitution of a protein-free diet for a complete diet in the rat.

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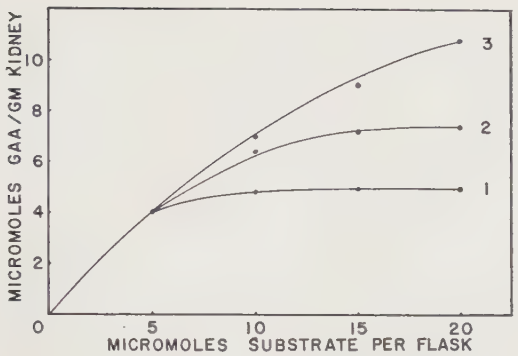

FIG. 1. Guanidinoacetic acid production as a function of varying concentrations of arginine and glycine. In series 1, arginine conc. was varied with constant glycine conc. of 5 μmoles/flask. Glycine was varied with arginine constant at 5 μmoles/flask in series 2. In series 3, equal molar amounts of arginine and glycine were used. The reaction flasks contained: 0.5 ml 0.067 M Sorensen phosphate buffer, pH 7.4; 0.5 ml 4% pork kidney homogenate in buffer (20 mg of wet kidney); and 0.5 ml arginine-glycine solution. Initial conc. in reaction flasks were: buffer, 0.067 M; pork kidney, 1.33%; and, in series one: glycine, 3.3×10^{-3}M, and arginine, (3.3, 6.7, 9.9 and 13.3) $\times 10^{-3}$M; in series 2: arginine, 3.3×10^{-3}M, and glycine, (3.3, 6.7, 9.9 and 13.3) $\times 10^{-3}$M; and in series 3: arginine and glycine each (3.3, 6.7, 9.9 and 13.3) $\times 10^{-3}$M. Incubation was for 1 hr at 37°. Guanidinoacetic acid was determined as under "Methods and materials."

Methods and materials. Canavanine was isolated from Jack bean meal by the method of Kitagawa(6), and also was purchased from Nutritional Biochemicals Corp. Dimercaprol was purchased from Hynson, Westcott, and Dunning, Inc., Baltimore, Md. A crude liver arginase preparation was used to remove arginine from the incubation mixture prior to guanidinoacetic acid analysis. Thirty g fresh rabbit liver was homogenized with 70 ml cold water in a Waring Blendor and the homogenate dialyzed against cold water for 2 days at 4°C. The homogenate was centrifuged in the cold for 15 minutes at approximately 1000 relative centrifugal force and the supernatant fluid decanted and saved. The preparation could be kept frozen for months without loss of activity and had neither transamidinase nor transmethylase(7) activity. All solutions used in the incubation procedure were made up with 0.067 M phosphate buffer, pH 7.4. The assay was as follows: 0.5 ml of 4% kidney homogenate was added to 0.5 ml 0.04 M glycine solution and 0.5 ml 0.04 M arginine solution in a 25 ml Erlenmeyer flask. Incubation was for 1 hour at 37°C in a Dubnoff type metabolic incubator(8) with an air phase over the flasks. Prior to and after the incubation, 0.5 ml aliquots of the incubation mixture were removed, placed in a 15 ml centrifuge tube, and heated in a boiling water bath 10 minutes. After cooling, 0.5 ml of the liver arginase preparation was added and the mixture incubated for 1½ hours at 37°C. Protein was removed by the addition of 2.0 ml 0.3 N $Ba(OH)_2$ in phthalate buffer, pH 5.0, and 2.0 ml 5% $ZnSO_4 \cdot 7H_2O$. Two ml of the filtrate was used for colorimetric determination of guanidinoacetic acid(9).

Development of the assay. Effects of varying concentrations of arginine and glycine on guanidinoacetic acid production are shown in Fig. 1. With a constant amount of glycine at 5 μmoles/flask (series 1) a maximum guanidinoacetic acid production of 5 μmoles/gram kidney was obtained with 10 μmoles of arginine. With arginine constant at 5 μmoles/flask (series 2) a maximum production of 7.5 μmoles guanidinoacetic acid/gram kidney was obtained with 15 μmoles of glycine. Maximum production of guanidinoacetic acid was obtained with equal molar amounts of arginine and glycine (series 3). There was no production of guanidinoacetic acid by the kidney homogenate without added substrate. The effect of incubation time at various equal molar concentrations of arginine and glycine on guanidinoacetic acid production is shown in Fig. 2. The enzyme was still active after 3 hours incubation. It was assumed that the enzyme was sufficiently saturated with substrate after one hour of incubation with an initial amount of 20 μmoles each of arginine and glycine/flask (series 3). Forty μmoles of arginine/flask was an amount too great to be removed conveniently by the arginase preparation for guanidinoacetic acid analysis. The optimum temperature for transamidination by kidney homogenate was found to be 47°C. The effect of varying amounts of pork, rat, and rabbit kidney homogenate upon guanidinoacetic acid production was determined. 0.25, 0.5, or 1.0 ml of 4% homogenate in the assay resulted in a constant production per g kidney and was 9, 26, and 3

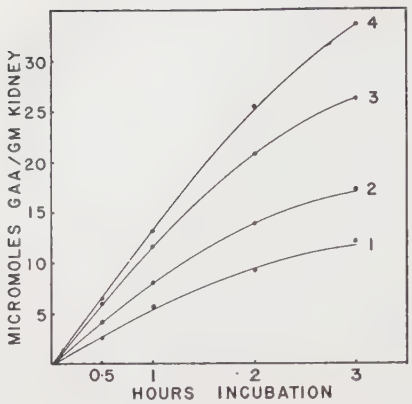

FIG. 2. Production of guanidinoacetic acid as a function of incubation time at different equal molar levels of arginine and glycine. Each flask contained 1 ml 0.067 M phosphate buffer, pH 7.4, 1 ml of 4% pork kidney homogenate, and 1 ml equimolar arginine-glycine solutions. Flasks 1, 2, 3, and 4 contained 4, 10, 20, and 40 μmoles each of arginine and glycine respectively. Initial conc. were: buffer, 0.067 M; pork kidney, 1.33%; and arginine and glycine each, (2.6, 6.7, 13.3 or 26.7) $\times$ 10^{-3}M. Incubation was at 37°C. Guanidinoacetic acid was determined as under "Methods and materials."

μmoles/hour for pig, rat, and rabbit respectively. Borsook and Dubnoff report by their procedure 4.6 and 3.4 μmoles/g kidney/hour for rabbit and rat respectively(1). Transamidinase activity was found only in kidneys. Twenty per cent homogenates of spleen, liver, blood, heart, muscle, testes, and brain from a normal rat had no activity. Borsook and Dubnoff also found no activity in these tissues.

Sulfhydryl inhibitors. 0.15 μmole of parachloromercuribenzoic acid produced complete inhibition of the system. One μmole of reduced glutathione, or 2 μmoles of cysteine, or 1 μmole of dimercaprol resulted in a 122, 105, and 78% reversal of the inhibition respectively. Addition of reduced glutathione to an uninhibited system resulted in a 138% production as compared to the control flask. Cysteine or dimercaprol did not increase production in the uninhibited system. These results indicated that transamidinase is a sulfhydryl enzyme(10). The *in vivo* inhibition studies were carried out on a 125 g albino rat. The rat was given a dose of 5 mg $HgCl_2$/kilo body weight by intramuscular injection of a solution containing 5 mg/ml in

TABLE I. Substitution of Canavanine for Arginine as Substrate for Transamidination.

μmoles substrate/flask			μmoles guanidinoacetic acid formed/g rat kidney/hr
Glycine	Arginine	Canavanine	
20		20	22
20	20		7
20	20	20	13
20	20	40	22

0.9% NaCl. Seventy-two hours after the injection the rat was anesthetized with ether and 5 ml of blood removed by heart puncture for creatinine and urea determinations. The kidneys were removed; one was preserved in formalin for morphological examination and one was used for the enzyme assay. The cortices were found to be white and the medulla was congested. The serum creatinine and urea nitrogen was found to be 3.8 mg% and 85 mg% respectively, which indicated severe renal damage. A transamidinase assay showed an activity of 2.7 units as compared with a normal activity of 25 units. The addition of dimercaprol to the reaction flask did not restore the transamidinase activity.

Substitution of canavanine for arginine as the substrate. Canavanine previously has been reported to be an amidine group donor for the transamidinase system(4,5,11). The data in Table I confirm these findings. Arginine was approximately ⅓ as effective as canavanine and appeared to inhibit canavanine as an amidine donor. The guanidinoacetic acid formed in these experiments was also identified by filter paper chromatography. Transamidination with canavanine as the substrate was found to be inhibited by PCMB and this inhibition was reversed with glutathione, cysteine, and dimercaprol as with arginine as the substrate.

Effect of protein-free diet on kidney transaminidase. Twelve weanling, albino rats were used to determine effect of a protein-free diet on kidney transaminidase. The complete diet consisted of: lactalbumin, 180 g, dl-methionine, 2.5 g, sucrose, 726.5 g, choline, 1 g, corn oil, 50 g, salt mixture(12), 40 g, and a vitamin preparation (Upjohn "Zymadrops"), 1.2 ml. The protein-free diet was the same except that lactalbumin was replaced by su-

TABLE II. Effect of a Protein-Free Diet on Kidney Transamidinase Activity of Weanling Rats.

Group*	Avg wt on day 0	12	27	Avg kidney Wt	% N	Units activity	Total activity	Activity per mg N
1	50	38		.3	2.5	3.9	1.1	.15
2	48	33	60	.4	2.6	13.5	5.4	.51
3	50	67	63	.4	2.9	3.4	1.4	.12
4	52	77		.5	2.7	17.5	8.8	.63

* Group 1 received the protein-free diet for 12 days. Group 2 received protein-free diet 12 days, followed by complete diet for 15 days. Group 3 received complete diet for 12 days, followed by protein-free diet for 15 days. Group 4 received a complete diet for 12 days. All animals were given food and water *ad libitum*. A unit activity is described as μmoles guanidinoacetic acid formed/g kidney (wet wt)/hr.

crose. Six weanling rats were fed the complete diet, and 6 were fed the protein-free diet for 12 days. On the twelfth day, 3 rats from each group were sacrificed and kidney transaminidase activities determined. A separate kidney homogenate was made for each rat, and an aliquot removed for micro-Kjeldahl nitrogen analysis. The dietary regimen of the remaining animals was then changed as follows: Rats which had been on the protein-free diet received the complete diet, and the rats which had been on the complete diet received the protein-free diet. Fifteen days later, the rats were sacrificed, and transaminidase and nitrogen analyses performed as above.

The results of this experiment are shown in Table II. Kidneys from rats fed a protein-free diet for 12 days had only 24% as much activity per mg kidney nitrogen as kidneys from rats fed the complete diet during the same period. Kidneys from rats fed a protein-free diet 12 days followed by a complete diet for 15 days had 80% as much activity per mg kidney nitrogen as kidneys from the rats fed the complete diet for 12 days. Kidneys from rats fed a complete diet for 12 days followed by a protein-free diet for 15 days had 19% as much activity per mg nitrogen as kidneys from rats fed the complete diet for 12 days. These results indicate that transaminidase activity of rat kidney is dependent on dietary protein intake. This is in marked contrast to kidney D-amino acid oxidase, which shows little or no change of activity in a protein depleted rat (13-16).

Summary and conclusions. 1. An assay of transaminidase activity in mammalian kidneys was developed. 2. The enzyme was inhibited by p-chloromercuribenzoic acid. The inhibition could be reversed with reduced glutathione, cysteine, or dimercaprol. These results suggest that transaminidase is a sulfhydryl enzyme. 3. Kidneys from a mercury-poisoned rat showed reduced transaminidase activity. The activity could not be restored by addition of dimercaprol to the reaction flask. 4. Canavanine was found to be 3 times as effective as arginine as an amidine donor in the transamidinase system. 5. Kidney transaminidase activity in weanling rats fed a protein-free diet for 12 days was 24% of normal. The activity was restored to 80% of normal by feeding the rats a complete diet for 15 days following the feeding of a protein-free diet for 12 days. Thus, kidney transaminidase activity appears to be dependent on exogenous protein sources.

The authors wish to thank F. Bollum for helpful suggestions concerning liver arginase.

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Cheyne-Stokes Breathing after Denervation of Carotid and Aortic Chemoreceptors and Sino-Aortic Pressoreceptors.* (23133)

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According to some theories concerning the mechanism of production of Cheyne-Stokes breathing oxygen-lack drive from chemoreceptors is a factor(1), while in other theories reflex drive is not mentioned(2). If Cheyne-Stokes breathing in any given instance is dependent upon a seesawing between the action of CO_2 on the respiratory center and oxygen lack drive exerted reflexly from chemoreceptors in the carotid and aortic bodies, it could not occur in animals having the chemoreceptors denervated, or if it should appear in an animal with IXth and Xth nerves intact it should be eliminated by denervation. In the course of studies on other aspects of control of respiration typical Cheyne-Stokes breathing was observed in 2 dogs having the sino-aortic zones denervated.

Methods and results. Each of the 2 dogs was anesthetized with sodium pentobarbital, 30 mg/kg, and had been bilaterally vagotomized at the upper cervical level. In the first experiment 500 ml of blood had been withdrawn from the dog which weighed 26 kg. Next, the region of the carotid bifurcation on each side was isolated by ligatures and excised. Shortly after this Cheyne-Stokes breathing developed. The respiratory record is shown in Fig. 1. Some of the apneic periods were as long as 40 seconds. The arrhythmia disappeared after a time to be replaced by rapid shallow breathing and returned after injection of an additional 5 mg of sodium pentobarbital per kg. The surgical procedures performed in this animal regularly result in elimination of the respiratory stimulation which is produced before denervation by 1 to 2 mg of NaCN injected intravenously; however, unfortunately, the cyanide test was not performed in this animal.

In the second dog (weighing 8 kg) the Cheyne-Stokes breathing appeared prior to isolation of the second one of the carotid zones. This animal showed a pronounced respiratory stimulation in response to 1 mg of NaCN, intravenously, before denervation. The carotid bodies and sinuses were isolated by means of ligatures placed on the appropriate arteries. The carotid arteries were cut open bilaterally to demonstrate that no blood supply to the bodies or sinuses remained. The Cheyne-Stokes respiration was a little more

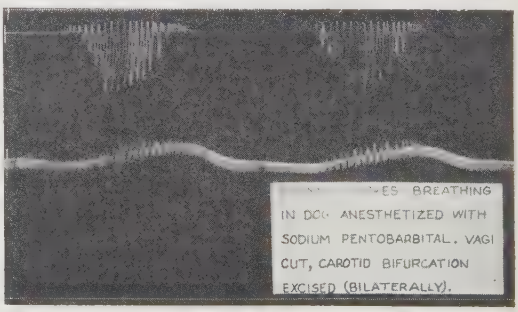

FIG. 1. Cheyne-Stokes breathing in dog having the vagi sectioned and carotid sinuses and bodies excised bilaterally. *Above:* Record of respiration. Downstroke indicates inspiration. *Below:* Blood pressure from femoral artery.

* Presented at meetings of Am. Physiol. Soc., Rochester, N. Y., Sept., 1956.

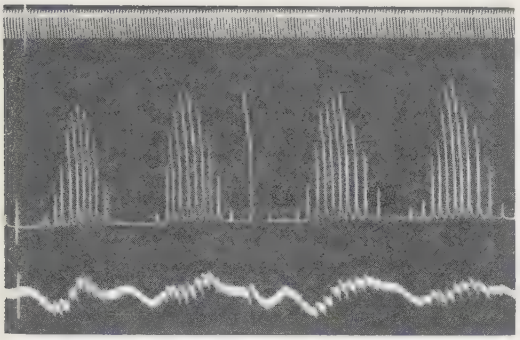

FIG. 2. Cheyne-Stokes breathing in dog after bilateral vagotomy and isolation of carotid sinuses and bodies. *Above:* Record of respiration. Upstroke indicates inspiration. *Below:* Blood pressure from femoral artery.

prominent after completion of the denervation. A record of the breathing at this stage is shown in Fig. 2. Shortly following this 1 mg of NaCN injected intravenously was found to have no effect on breathing. It must be concluded that oxygen-lack drive from the chemoreceptors was not a factor in the production of the Cheyne-Stokes breathing which developed under our conditions. Also, since sino-aortic pressoreceptors are isolated or denervated by the same procedures, rhythmic alterations in pressure in the sino-aortic zones are not concerned in the Cheyne-Stokes breathing observed.

Although the Cheyne-Stokes breathing illustrated here was independent of influences from carotid and aortic chemoreceptors and sino-aortic pressoreceptors, it still is possible that a similar type of irregularity can be produced by an imbalance of central and reflex drive. Several facts lead one to believe that Cheyne-Stokes breathing may be produced by 2 or more different mechanisms. For example, the mean arterial blood pressure shows a cyclic rise and fall during Cheyne-Stokes breathing, but in some instances the rise in blood pressure occurs during the apneic phase and in other instances the rise is during the hyperpneic phase. In both of the records shown here the latter type is seen. Possibly this type of periodic breathing has a purely central basis, while in the other type oxygen-lack drive from chemoreceptors may be a factor.

Summary. Typical Cheyne-Stokes breathing has been observed in 2 dogs having the vagi sectioned bilaterally at the upper cervical level (denervating aortic bodies and aortic pressoreceptors) and having the carotid bodies and sinuses isolated or excised bilaterally. Therefore, neither oxygen-lack drive from chemoreceptors nor reflex effects on breathing from rhythmic fluctuations in pressure in the sino-aortic zones are concerned invariably in the production of Cheyne-Stokes breathing.

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Effect of Beta-Aminopropionitrile on Reproduction of Chickens.* (23134)

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Diets containing *Lathyrus odoratus* seeds or beta-aminopropionitrile (BAPN) cause extreme abnormalities of the skeleton, hernias, dissecting aneurysms of the aorta and reproductive failure in rats(1-4). In turkeys, which are more sensitive to BAPN(5) than rats, nerve damage, internal hemorrhages and hock deformities are observed with only slight abnormality of the skeleton. Skeletal deformities were the outstanding effect observed in rats which indicates that BAPN interferes with calcium metabolism. The high level of calcium metabolism of the laying hen suggested a study of the susceptibility of this animal to BAPN toxicity.

* Technical Contribution No. 267, S. Carolina Agri. Exp. Station. Published by permission of Director.

TABLE I. Basal Ration.

	%
Ground yellow corn	60
" wheat	10
Soybean oil meal (44% protein)	10
Alfalfa meal	5
Dried whey	3
Fish meal	2.5
Meat scraps	"
Vitamin and UGF supplement*	"
Defluorinated rock phosphate	3.0
Ground limestone	1.0
Salt	.3
Manganese sulfate	.025
Vitamin A	498 USP units/lb
" D	205 ICU/lb

* Contains sardine fish meal, condensed fish solubles, whey solubles, hydrolyzed cod livers, active dry yeast, grass juice concentrate, penicillin mycelium meal, riboflavin feed supplement, B_{12} feed supplement, niacin, choline chloride and calcium pantothenate. Guaranteed analysis in mg/lb: riboflavin 44, B_{12} 0.3, niacin 500, choline 4,500 and pantothenic acid 50.

Methods. Single Comb White Leghorn hens were distributed at random into 5 groups each containing 6 birds. They were maintained in laying batteries with raised wire floors. Feed, water and oyster shells were supplied *ad libitum*. All birds received the basal diet (Table I) for 3 weeks. Each of 3 groups were then fed the basal diet with 0.01%, 0.03% or 0.06% added mono-beta-aminopropionitrile • fumarate (BAPN) for 4 weeks followed by the basal diet for 3 weeks. Two groups received the basal diet throughout the 10-week period. All birds were artificially inseminated weekly with 0.1 cc pooled semen. Eggs were set at weekly intervals and were candled after one week of incubation. Those eggs not containing live embryos were broken out and examined carefully to distinguish infertiles from early deaths. All dead embryos were carefully examined for determination of approximate time of death and gross abnormalities.

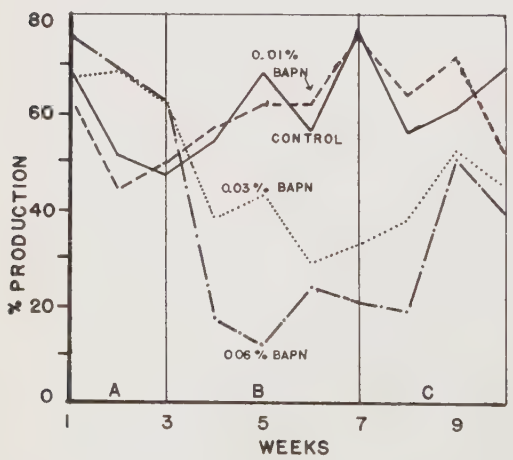

FIG. 1. Effect of BAPN on egg production. During period A all birds received basal diet. Experimental diets were fed during period B and all birds were returned to the basal diet during period C.

Results. The average *egg production* of the 2 control groups fluctuated from a low of 47% at the beginning of the supplemental period to a high of 77% at the end of this period (Fig. 1). The birds fed 0.01% BAPN laid at the rate of 50% the week before its addition and reached 76% production at the end of 4 weeks supplementation.

The week prior to addition of 0.03% BAPN to the diet those birds laid at the rate of 62%. During the 4-week supplemental period they reached a low of 29% production. The birds which received 0.06% BAPN dropped from 62% pre-supplemental to a low of 12% during the supplemental period. Both groups increased their rate of production after removal of BAPN although they did not reach their previous level in the 3-week post-supplemental period.

Shortly after BAPN was administered to certain groups *soft shelled and malformed eggs* were laid by those birds consuming .03% and .06% of this material. The incidence of soft shelled eggs increased until 33% of all eggs laid were soft shelled in the group fed .03% BAPN, and 78% of all eggs laid were soft shelled in the group receiving .06% BAPN (Table II). Malformations included

TABLE II. Effect of BAPN* on Incidence of Soft Shelled Eggs.

% BAPN added during supplemental period	% of eggs laid which were soft shelled									
	Basal period			Supplemental period				Basal period		
	Weeks									
	1	2	3	4	5	6	7	8	9	10
.0	0	0	0	0	0	0	6	14	3	3
.0	0	0	0	0	0	0	7	13	14	9
.01	0	0	0	0	0	0	0	4	3	14
.03	0	0	0	6	11	25	33	13	0	0
.06	0	0	0	0	0	60	78	38	14	12

* BAPN in the form of mono-beta-aminopropionitrile • fumarate.

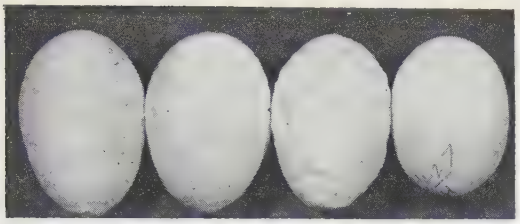

FIG. 2. Malformed eggs caused by feeding BAPN at .03% or .06% of the diet.

wrinkled, checked and ridged eggs (Fig. 2). BAPN fed at the level of .01% did not affect shell formation.

Hatchability of fertile eggs of the control groups varied from a high of 91% the second week of the experimental period to a low of 61% at the end of the experimental period (Fig. 3). The group receiving .01% BAPN dropped from 92% hatchability prior to treatment to 41% at the end of the experimental period. The birds supplemented with .03% dropped from 65% hatchability to 0% at the end of the experimental period and the group receiving 0.06% dropped from 84% to 0% after three weeks of BAPN supplementation. The hatchability immediately improved in all groups following the withdrawal of BAPN. It should be pointed out that the poor rate of production and the high incidence of soft shelled and malformed eggs reduced the number of eggs incubated to 4 or 5 fertile eggs in some instances and to no fertile eggs on the fifth and seventh setting at the highest level of supplementation. The hatchability of the control group was based on settings of no less than 26 fertile eggs.

Discussion. Addition of .01% BAPN to the diet of the laying hen appeared to have no detrimental effect on egg production or shell formation. Levels of .03% and .06% BAPN reduced rate of production and induced a high percentage of soft shelled and malformed eggs. BAPN at .01% reduced hatchability to 41% while .03% and .06% BAPN caused hatchability to drop to 0 in 3 or 4 weeks. Hatchability promptly returned to a satisfactory level following removal of the toxic compound from the diet of the birds.

The effect of BAPN on shell formation suggests that hatchability might be reduced because of poor shell formation. Examination of the dead embryos, however, revealed that 40% to 80% of the embryonic mortality on the .03% and .06% levels of BAPN was due to hemorrhages occurring primarily in the 3rd week of incubation. These figures are based on a small number of observations but they suggest that embryonic mortality is not a mere reflection of poor shell quality but is due in part at least to degeneration of the circulatory system as observed in rats(4) and turkeys(5). The ability of BAPN to inhibit shell formation suggests its use in further elucidating the mechanism of shell formation and indirectly in studying its method of interference with calcium metabolism of rats.

Summary. Mono-beta-aminopropionitrile • fumarate (BAPN) at .03% and .06% of the diet of laying hens reduced egg production from 62% to 29% at the lower level and from 62% to 12% at the higher level of supplementation. These levels of supplementation caused a high incidence of soft shelled and malformed eggs. After 4 weeks supplementation 33% of all eggs laid were soft shelled at the .03% dietary level of BAPN and 78% were soft shelled at the .06% level. BAPN at the level of .01% of the diet did not affect egg production or shell formation. Hatch-

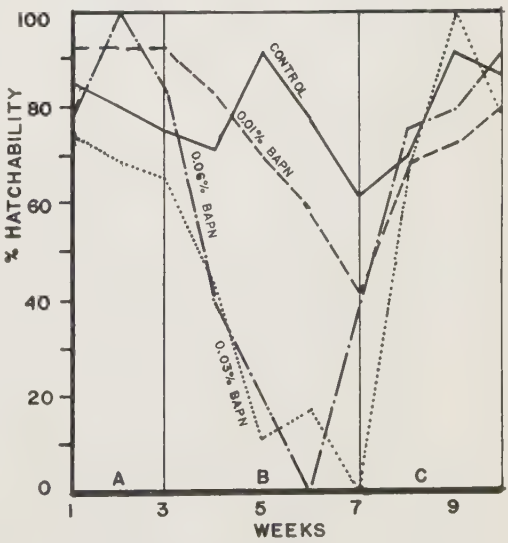

FIG. 3. Effect of BAPN on hatchability of fertile eggs. During period A all birds received basal diet. Experimental diets were fed during period B and all birds were returned to basal diet during period C. There were no fertile eggs set during week 5 or 7 at the .06% level.

ability of fertile eggs was decreased by levels of .01%, .03% and .06% of BAPN in the diet. Hatchability was reduced to 41% in the group receiving .01% BAPN after 4 weeks supplementation compared to the control at 61%. Hatchability decreased to 0 in 4 weeks at the .03% level and to 0 in 3 weeks in the group receiving .06% BAPN. Following removal of BAPN from the diet both egg production and hatchability increased and the percent of soft shelled eggs decreased. Hemorrhages were found in 40% to 80% of the dead embryos from the groups fed .03% and .06% BAPN.

The authors are grateful to Abbott Laboratories, N. Chicago, Ill., for supplying the beta-aminopropionitrile • fumarate.

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Secondary Shock in the Dog Produced by Total and Partial Aortic Occlusion.* (23135)

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Secondary shock is typically characterized by a reduced cardiac output, arterial hypotension and generalized stagnant anoxia. The result is usually a marked hypofunction of internal organs which if continued leads to death of the organism. Of the vital processes and organs which fail in irreversible shock, it is not known which failures are primary and which secondary, or conversely which systems or organs if protected from the anoxia would exert maximum survival benefit. There is reason to believe that increased perfusion of the liver(1,2) will lower the mortality in experimental shock but evidence of this type with reference to other organs or systems is scarce. It would seem that a method of producing experimental shock which permitted graded and controlled degrees of anoxia of various parts of the body might be helpful in this problem; regulation of degree of arterial flow appears to be one of the best means of achieving this. Aortic occlusion shock was produced by Erlanger and Gasser(3) but this method has since been little used.

The object of the present study was a) to determine whether repeatable experimental shock could be induced by total or partial aortic occlusion and b) to determine whether such a method might be effective in producing different degrees of anoxia in different parts of the body. The ultimate aim was to study the relative importance of various systems and organs in the genesis of fatal shock.

Methods. Mongrel dogs were anesthetized with morphine sulfate (3 mg/kg) and intravenous sodium barbital (120 to 180 mg/kg). Blood pressures were recorded with damped mercury manometers. Temporary occlusion of the lower thoracic aorta (Th 8-9) was produced either by careful tightening of a polyethylene noose placed surgically 2 to 3 weeks before, or by inflation of an intraaortic balloon catheter containing radioopaque liquid; the catheter was passed via the femoral or carotid artery under fluoroscopic guidance. The two methods yielded comparable results but the balloon catheter was used almost ex-

* This investigation was supported by Research and Development Division, Office of Surgeon General, Department of Army under Research Contract.

TABLE I. Results of Temporary Occlusion of Lower Thoracic Aorta in Dogs (Th 8-9).

Group	Type of occlusion	Occl. time (hr)	No. of dogs	Mean wt (kg)	No. died	% mort.	Mean survival time (hr)
A	Total	1½ to 2	9	15.2	9	100	10.5
B	Partial	2 to 2½	8	15.3	3	37.5	45.3

clusively after early experiments because of its greater simplicity and ease of control. The volume of the balloon was regulated by means of a threaded shaft and piston attached to the syringe plunger; in this way degree of aortic occlusion and thus distal aortic pressure could be accurately controlled. Total and partial thoracic aortic occlusions were produced for periods ranging from 1½ to 2½ hours. In some experiments, occlusions resulting in a mean distal aortic pressure of 30 mm Hg were combined with simultaneous controlled bleeding from a carotid artery into an inverted reservoir containing Mepesulfate† in saline (200 mg %). These latter animals were therefore subjected to shock in which the mean aortic perfusion pressure above the diaphragm was 100 mm Hg and that below was 30 mm Hg. At the end of the hypotensive period the balloon was removed, the withdrawn blood reinfused and the wounds closed. In a number of animals, degree of oxygenation of the liver and lower segments of the body was studied by determination of blood oxygen content and capacity of arterial, hepatic venous and inferior vena caval blood before, during and after occlusion, using the methods of Van Slyke(4) and Roughton(5) for blood oxygen. Hepatic venous samples were obtained from a right hepatic vein via cardiac catheter; inferior vena cava samples were taken from a point 2 to 3 inches below the liver.

Results. In 2 groups of animals (Group A and B), total and partial occlusions of the lower thoracic aorta at levels of Th 8-9 were produced for periods of 1½ to 2½ hours. In the partial occlusions most of the animals were subjected to mean distal aortic pressures of either 40 or 30 mm for periods of 2 hours. The general results are summarized in Table I.

† The anticoagulant Mepesulfate was kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

During the occlusion the skin temperature of the lower half of the body decreased markedly, respiratory rate and depth was diminished, particularly in the animals in Group B, and bloody diarrhea often supervened. In Group A post-occlusion blood pressures were often markedly decreased from the control values and most of these animals died in coma within 12 hours after release of the balloon. At autopsy these dogs had considerable amounts of bloody fluid in the large and small intestine but relatively little gross congestion of the mucosal and serosal vessels of these organs. The lungs were wet and severely congested.

Both of these types of experiments had certain disadvantages—in the total occlusion the stress was too severe and in the partial occlusions the central hypertension and pulmonary congestion were undesirable concomitants.

In Group C a combined hemorrhagic aortic-occlusion stress was produced by occluding the aorta to a distal arterial pressure of 30 mm Hg while simultaneously bleeding the central portion of the arterial bed to a fixed hypotensive level of 100 mm Hg. The aortic occlusion was produced 2 to 3 inches above the coeliac axis artery (Th 8-9) so that a severe arterial hypotension existed throughout the subdiaphragmatic aorta and its branches. As controls, Group D animals were subjected only to hemorrhagic hypotension of 100 mm; in these experiments the balloon catheter was inserted but not inflated. The results are shown in Table II and the blood pressure responses of all the groups are summarized in Table III.

To obtain a measure of the effect of these procedures on state of oxygenation of the involved tissues, the oxygen content and capacity of hepatic venous and inferior vena caval blood were determined in some of the animals. As measurements of the state of oxygenation, such values are by no means

TABLE II. Summary of Combined Hemorrhagic-Aortic Occlusion Shock.

Group	Type of shock	Duration of shock (hr)	No. of animals	Mean body wt (kg)	No. died	% mort.	Mean survival time (hr)
C	Hemo + aortic occl. (100-30 shock)	2½	12	10.5	10	83.3	13.1
D	Hemo only (100 mm shock)	2½	12	12.9	1	8.3	34.0*

* Single animal.

TABLE III. Blood Pressure Responses of Dogs Subjected to Occlusion of Lower Thoracic Aorta (Th 8-9).

Group	No. of animals	Type of occlusion	Mean arterial blood pressures (mm Hg)			
			Before	During occlusion† Carotid	Femoral	After
A	9	Total	113.7 ± 24.0*	173.4 ± 16.7	14.7 ± 2.9	88.3 ± 32.1
B	8	Partial	121.8 ± 21.8	181.6 ± 15.4	35	124.1 ± 30.1
C	12	Partial + hemo.	117.3 ± 17.2	100	30	113.7 ± 14.7
D	12	None—hemo. only	125.5 ± 18.1	100	100	126.3 ± 18.3

* Refers to stand. dev. of distribution.
† Representative pressures taken at midpoint of occlusion period.

exact indices; the saturation of venous blood draining a part however is undoubtedly a reasonable function of the tissue pO_2 and as such represents the summated effect of both the oxygen supply and oxygen utilization of the part. The venous saturation values are given in Table IV.

Discussion. Irreversible shock produced by the combined hemorrhage-aortic occlusion technic described above resembles experimental hemorrhagic shock in many respects. During the stress there is a decrease in body temperature, increase in heart rate, depression of reflexes and tendency to 'take up' blood from the reservoir. Following release of the occlusion and reinfusion of the withdrawn blood the arterial blood pressure returns to control levels or above, heart rate declines and circulation through the occluded areas returns to approximately normal. Over the following 6 to 24 hours blood pressure gradually declines, bloody diarrhea often occurs and death supervenes in about 80% of cases. One of the more notable contrasts with hemorrhagic shock is the lack of the usual accelerated respiratory rate and air hunger; instead, the animals in hemorrhage-aortic occlusion shock showed a decrease in respiratory rate to levels of 2 to 6 per minute. This was undoubtedly due to increased arterial flow to the head region.

The oxygen saturations of venous blood below the occlusion point varied considerably from animal to animal but did reveal the unmistakable and drastic degree of stagnant anoxia which prevailed particularly in the liver. The degree of hepatic anoxia was closely comparable to that previously reported

TABLE IV. Oxyhemoglobin Saturation of Venous Blood in Aortic Occlusion Shock.

Group	Type of shock (No. of animals)	Mean HbO_2 saturations (%)					
		Hepatic vein			Inf. vena cava*		
		Before	During	After	Before	During	After
A	Total aortic occl. (5)	56.9 ± 10.68	5.1 ± 7.44	44.3 ± 17.92	74.1 ± 4.79	31.4 ± 14.06	56.2 ±16.32
C	Hemo. + partial occl. (5)	75.7 ± 10.06	6.8 ± 7.61	77.4 ± 7.87	83.4 ± 3.78	27.0 ± 17.26	79.5 ± 9.27
D	Hemo. only—to 100 mm Hg (6)	76.4 ± 5.69	38.9 ±24.88	62.0 ± 10.58	82.7 ± 7.16	69.8 ± 10.84	74.6 ± 9.15

* Sampling done below liver. ± refers to stand. dev. of distribution.

in hemorrhagic shock in the dog(6).

The results indicate that hemorrhage-aortic occlusion results in a repeatable type of experimental shock in the dog which simulates pure hemorrhagic shock in most of the important essentials. The method permits simultaneous and independent control of arterial perfusion pressures to different segments of the body. While it has the advantage of achieving differential perfusion without the necessity of extensive surgery or extracorporeal circulation, its application is somewhat limited by the anatomical vagaries of the arterial supply of the involved organs.

Summary. 1) A combination of hemorrhagic and partial aortic occlusion produces a controllable degree of hypotensive shock in the dog which closely resembles hemorrhagic shock. 2) The method provides an opportunity to study the effects of differential arterial perfusion of different body segments.

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Effect of X Irradiation on Increase in Rat Liver Tryptophan Peroxidase Produced by Adrenal Steroids.* (23136)

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On the basis of measurements of liver glycogen and blood glucose in X-irradiated rats, Mole has recently taken issue with the concept that irradiation stimulates the adrenals (1). He suggested that the failure of X-irradiated adrenalectomized rats to synthesize glycogen is due not directly to lack of cortical hormones but rather to a low level of blood glucose, below the threshold for glycogen deposition; and that glycogen synthesis following administration of cortisone is the result of an increase in concentration of glucose in blood to a level favoring deposition of glycogen. Mole concludes that X irradiation has not been shown to increase adrenal activity, and that the irradiated rat actually uses cortisone more efficiently than controls.

One change which takes place in irradiated rats is an increase in tryptophan peroxidase activity of the liver(2). This increase does not occur in adrenalectomized rats, and hence resembles the increases produced by a number of amino acids and pharmacodynamically active agents(2,3,4), rather than the increase produced by tryptophan, which is considerably greater and is not prevented by adrenalectomy(3). However, the level of the enzyme can be increased in adrenalectomized rats by administration of cortisone or hydrocortisone, the response being roughly proportional to the dose over an appreciable range (5). We felt that this system would be suitable for studying any possible improvement in efficiency of utilization of the glucosteroids in irradiated animals.

Methods. Female rats of the Sprague-Dawley strain, 6 weeks old, were adrenalectomized 5 days before irradiation; during this time they received 1% saline *ad libitum,* and were injected with desoxycorticosterone acetate (1 mg/kg) on alternate days. Exposure to X rays was at the rate of 200 r/min from a machine operated at 250 kv, 15 ma, 0.5 mm Cu plus 3 mm bakelite filters, 27 cm distance. A dose of 600 r total-body irradiation was used throughout. Cortisone acetate (20 mg/

* Work performed under the auspices of the U. S. Atomic Energy Commission.

TABLE I. Effect of X-Irradiation and Hormone Injection on Tryptophan Peroxidase Activity of Livers of Adrenalectomized Rats.

	Kynurenine formed, μM/hr/g dry wt								
	Non-irradiated			600 r, inj. immediately			600 r, inj. after 24 hr		
Treatment	No.	Avg	σ	No.	Avg	σ	No.	Avg	σ
No inj.	23	7.4	1.9	4	7.9	.5	8	6.7	1.0
Hydrocortisone, 3 mg/kg	5	21.0	3.4	5	16.6	1.7	5	17.6	4.9
Cortisone, 20 mg/kg	5	19.6	3.9	5	14.3	2.4	5	16.1	4.5

kg) and hydrocortisone acetate (3 mg/kg) were given intramuscularly as saline suspensions either 0 or 24 hours after irradiation. Six hours later the rats were killed, and the livers rapidly excised and frozen between blocks of solid CO_2. This treatment did not affect enzyme activity. Tryptophan peroxidase was measured by the method of Knox (3) on homogenates prepared from the frozen tissues.

Results. Table I shows that irradiation did not augment response of adrenalectomized rats to either of the steroids administered. The decreases observed in the irradiated rats are not statistically significant, although they are of interest. As reported earlier, hydrocortisone was about 7 times as active as cortisone(5) in both irradiated and non-irradiated animals.

Discussion. These data fail to demonstrate any increase in the efficiency of utilization of cortisone or hydrocortisone by the irradiated rat so far as an increase in liver tryptophan peroxidase was concerned; if anything, the response to glucosteroid injection was slightly decreased. The response of the enzyme to administration of tryptophan has similarly been shown to be unaffected by radiation, at least during the first 3 to 4 days after exposure(2,6).

Knox and Auerbach, discussing the hormone- and substrate-induced adaptations of tryptophan peroxidase, attribute the increase in activity following administration of various compounds (histidine, epinephrine, histamine, etc.) to the effect of glucosteroids released either by direct action on the adrenals or by action of ACTH(7). Radiation also belongs in this category. The actual mechanism by which X irradiation stimulates the adrenals, as manifested by the increase in liver tryptophan peroxidase, is not known; however, it may be pointed out that maximum activity of the enzyme occurs within a few hours after initial release of histamine. There are doubtless other compounds liberated within a few hours after irradiation as a result of cellular destruction which could ultimately effect a rise in tryptophan peroxidase *via* stimulation of the adrenals.

Summary. On the basis of effectiveness in increasing tryptophan peroxidase activity of the liver, there was no evidence for an increased utilization of cortisone or hydrocortisone by X-irradiated adrenalectomized rats.

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Serum Alkaline Phosphatase in Dogs with Experimental Splenic and Renal Infarcts and with Endocarditis. (23137)

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In studies of experimental endocarditis in dogs(1), infarcts of kidney and spleen were encountered frequently. Since these organs contain abundant alkaline phosphatase, and since the enzyme was depleted in the infarcted areas, studies were made on the serum alkaline phosphatase values. To aid in interpretation of these data, studies of alkaline phosphatase were made also on dogs with bland infarcts produced by vascular ligation. As will be shown, the development of infarcts of kidney and spleen, whether produced by vascular ligation or by emboli in the course of bacterial endocarditis, may be indicated by a sharp rise in serum alkaline phosphatase.

Materials and methods. Eighteen mongrel dogs of both sexes weighing 10 to 15 kg were selected, after quarantine at our animal hospital. Infarcts of the spleen were produced in 6 dogs by tying off multiple branches of the splenic artery at the hilus. Infarcts of the kidney were produced in 7 dogs by tying off several branches of the left renal artery at the hilus (dog 13, Table I), the left renal artery (dogs 1, 4, and 16), or the left renal artery and vein (dogs 5, 15, and 18). The renal vein was ligated to determine if this procedure would alter the elevation of serum alkaline phosphatase, perhaps by slowing absorption of the enzyme from the infarct. In 5 dogs used as controls, an exploratory laparotomy was performed, but vessels to the spleen and kidney were not ligated. Blood for determination of serum alkaline phosphatase values was obtained before (usually within an hour) and at intervals after sur-

TABLE I. Serum Alkaline Phosphatase in Bodansky Units in Operated Control Dogs and in Dogs with Infarcts following Ligation of Splenic and Renal Vessels.

Dog	Preop.*	Immed. postop.†	Days after operation 1‡	2	3	4	5	6	7	9	10	11
Operated control dogs												
6	2.0	.4	2.7	2.4	2.9	2.9		2.9				
8	1.0	.7	.3	1.4	1.4	1.4	1.1		.7			
9	.5	1.2	1.0	1.3	1.2	1.2	.9					
12	1.8		.3	.1	.8	.8	.7			.3		
14	1.3	1.3	2.7	1.5	2.2	2.2	2.0		1.3			
Dogs with splenic infarcts												
2	.7		5.4	6.0	4.7	3.5	2.8			1.3		.6
3	1.1	1.3	2.1	2.2	1.5				.0			
7	3.4	3.6	3.5	3.7	3.9	4.6			3.5			
10	.4	1.2	1.1	1.1	1.1	1.3			1.2			
11	1.8		3.4	3.5	2.2	2.9	2.0			2.3	1.7	
17	.9		3.4	3.1	2.2			1.7				
Dogs with renal infarcts												
1	.2		6.8	6.2	2.7	3.3	1.7			1.2		1.8
4	.5	.5	6.9	4.9	3.2				2.0	1.5		
5	1.5	0	4.6	5.0	3.9			1.6				
13	.6		4.0	4.0	3.8	3.2	2.4			.7	1.4	
15	.2	.4	1.2	.9	.4	.4			.0			
16	3.0	2.1	5.6	4.8	2.6			2.6	2.4			
18	3.0	.8	5.7	13.5	12.1				10.1	6.4		

* Blood usually taken 1 hr before surgery. Earlier additional samples, taken in some dogs, showed similar values.

† Blood taken 3 to 6 hr after surgery except 1 hr for dog 16.

‡ " " 18 to 24 " " " .

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TABLE II. Serum Alkaline Phosphatase in Bodansky Units in Dogs with Aortic Insufficiency before and after Inducing Endocarditis by Intravenous Bacterial Injections.

Dog	Before* inj. bact. alk. phos.	After inj. bacteria: Days†	After inj. bacteria: Alk. phos.	Postmortem findings: Days†	Postmortem findings: Infarcts‡
Dogs given *Streptococcus mitis*					
63	1.1	5	3.7	7	R
		7	4.4		
66	1.7	7	2.2	11	R
67	1.3	6	4.2	11	R
		11	3.5		
108	.8	6	3.1	37	R, O
		14	1.6		
		20	1.1		
		28	1.5		
		35	7.6		
Dogs given *Staphylococcus aureus*					
71	3.2	4	6.4	6	R
		6	5.9		
114	.5	2	5.7	4	R
Dogs given *Staphylococcus aureus* and antibiotic therapy					
74	1.0	21	.8	37	—
70	3.0	7	8.0	17	O
78	2.4	4	2.3	28	O
		11	1.3		
		17	1.3		
75	1.0	11	4.5	28	O
		13	5.3		
		17	2.9		
		27	1.5		

* Blood for alkaline phosphatase was usually taken one to several days before inj. bacteria; earlier additional samples, taken in some dogs, showed similar values.

† Days after first bacterial inj.

‡ Infarcts are designated R if recent and O if they appeared to be older than 1 wk. Dog 70 had no infarcts and 78 had only a renal infarct; all others had renal and splenic infarcts.

gery (Table I). Values were determined with the aid of a Leitz photrometer using the methods of Fiske and Subbarow and of Bodansky(2). We found serum alkaline phosphatase in normal dogs to be under 2 Bodansky units, in most instances, and rarely above 3.5 units. Preliminary studies showed that, while serum alkaline phosphatase values may vary considerably in 2 different dogs, daily fluctuation in values in the same healthy dog is not great (see values after 2 days in controls, Table I). Dogs were sacrificed 7 to 12 days after surgery. Blocks of tissue from the infarcted organs were fixed in 65% ethanol and modification of the Gomori method(3) was used to demonstrate alkaline phosphatase. Other blocks were fixed in 10% buffered (pH 7.0) aqueous formalin and stained routinely with hematoxylin and eosin.

Dogs with aortic insufficiency have proved highly susceptible to endocarditis(1). Bacterial endocarditis was produced consistently in more than 80 such dogs by single or multiple intravenous injections of cultures of *Staphylococcus aureus* or *Streptococcus mitis*. The insufficiency was induced, usually about 2 weeks before injecting bacteria, by perforating an aortic leaflet with a punch(5). The endocarditis was more fulminating in dogs given staphylococci, but was prevented, arrested, or modified in some by early or delayed treatment with antibiotics (Table II). Determinations of the alkaline phosphatase level in the blood of 44 of these dogs were made at various intervals, particularly when certain clinical features such as increasing malaise and fever suggested possible infarction. The findings in 10 of these dogs, selected as a representative sample, are recorded in Table II.

Results. Postmortem studies revealed infarcts of the spleen (Fig. 1) or kidney (Fig. 2) in all the operated dogs except the controls. Since the dog kidney receives some blood from the ureteral artery, capsular vessels, and, in the female, from the ovarian artery(4), renal infarction was incomplete even after ligation of the renal artery and vein.

As shown in Table I, there was a significant elevation in serum alkaline phosphatase in 3 of the 6 dogs with splenic infarcts (dogs 2, 11, and 17), in 6 of the 7 with renal infarcts, but in none of the operated controls. The elevation developed within 24 hours and gradually dropped to normal in about a week. Some dogs showed a drop in values immediately after surgery. After ligation of both renal artery and vein, the enzyme level did not rise significantly in dog 15 (male), but was elevated in dogs 5 (female) and 18 (male).

Histologic studies revealed absence of alkaline phosphatase in the infarcted areas, except

Fig. 1

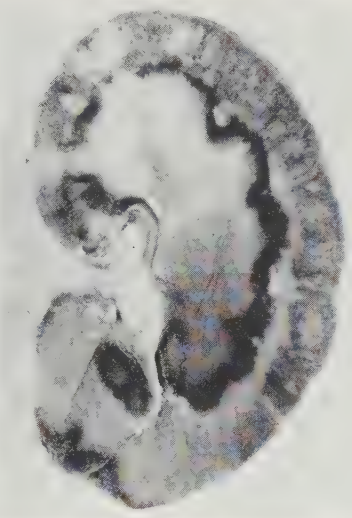

Fig. 2

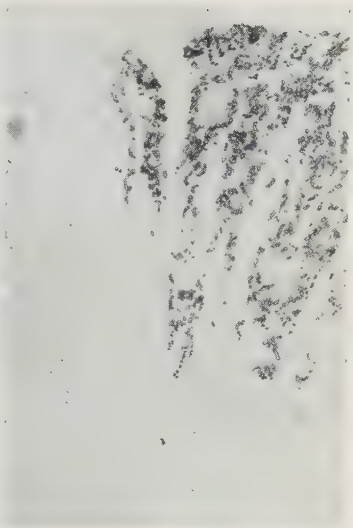

Fig. 3

FIG. 1. Infarcts in broad portion of spleen of dog 11 produced by ligating branches of the left splenic artery at the hilus.

FIG. 2. Infarcts of left kidney of dog 5 following ligation of left renal artery and vein.

FIG. 3. Kidney of dog 16 showing loss of alkaline phosphatase (black) in infarct on left. Alkaline phosphatase stained by modified Gomori method. × 10.

in occasional renal tubules (Fig. 3). Occasional tubules contained casts and debris reacting like alkaline phosphatase. The left kidney of dog 15, which did not develop an elevated serum alkaline phosphatase, nevertheless showed a massive infarct which was depleted of alkaline phosphatase.

In the endocarditis studies, nearly all the untreated dogs developed an elevation in serum alkaline phosphatase and died with endocarditis and multiple septic infarcts of the kidney and spleen (Fig. 4). The age of the infarcts was estimated by their gross and microscopic appearance(4) and corresponded closely with the estimated age based on a beginning rise in serum alkaline phosphatase. There were only occasional dogs with infarcts, illustrated by dog 66, Table II, in which no significant elevation in serum alkaline phosphatase was detected. The infection was prevented in some dogs by early antibiotic treatment. These dogs, illustrated by dog 74, did not develop a rise in serum alkaline phosphatase and did not show infarcts at autopsy. Therapy modified the course of endocarditis in others such as dogs 70 and 78 (Table II). Dog 78, which developed no apparent elevation in serum alkaline phosphatase, had only one small depressed yellow infarct in the right kidney. Generally, however, the development of infarcts in dogs receiving treat-

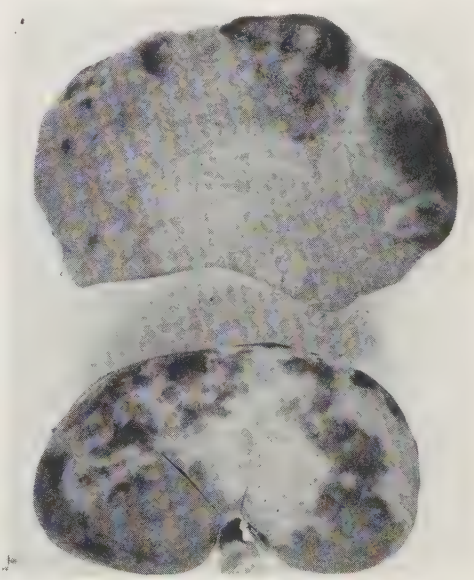

FIG. 4. Old pale yellow and recent dark red infarcts of spleen and kidney of dog 108 (Table II) which died 37 days after the first of 4 inj. of *Streptococcus mitis*.

ment was indicated by a rise in alkaline phosphatase and confirmed at autopsy (dogs 75 and 78, Table II).

Discussion. Our findings in operated dogs show that infarcts in spleen and kidney may cause a significant elevation in serum alkaline phosphatase level. The elevation is considered to be due to absorption of the enzyme from the infarcted areas. This elevation may aid in diagnosis of such infarcts and in following their course. The absence of such an increase, however, does not completely rule out the presence of a renal or splenic infarct. In our studies, some animals with splenic and occasionally with renal infarcts, such as dog 15 in Table I and 66 in Table II, failed to develop an elevation in serum alkaline phosphatase. To determine the causes of these failures requires further investigation.

We have found serum alkaline phosphatase values most useful in experimental endocarditis, in determining during life the occurrence of infarcts and their course. The value of serum alkaline phosphatase studies in man, in endocarditis and in the differential diagnosis of infarcts of spleen or kidney has not been determined.

Summary and conclusions. Infarcts of spleen and kidney were produced by vascular ligation in 13 dogs. The level of serum alkaline phosphatase increased significantly in 3 of 6 dogs with splenic infarcts and in 6 of 7 with renal infarcts. The level reached a maximum in about 24 hours and gradually returned to normal in about a week. An increase in serum alkaline phosphatase level was noted also in dogs with experimental endocarditis. This increase correlated well with the occurrence of infarcts in spleen and kidneys and proved to be of great diagnostic and prognostic value.

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Effect of Beta-Propiolactone on Complement-Fixing Antigens of St. Louis Encephalitis Virus.* (23138)

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A number of laboratory-acquired infections have occurred among individuals processing antigens for use in diagnosis of virus diseases (1,2). The only reported laboratory infection with St. Louis Encephalitis virus(3) occurred in an individual who was preparing complement-fixation antigen by lyophilization and extraction with benzene using the procedure described by Espana and Hammon(4, 5). More recently, a fatal infection with the virus of Russian Spring-Summer Encephalitis occurred as a result of an accident while processing antigens with the same procedure (6). An inapparent infection occurred in a laboratory worker who was working at the same laboratory bench. The present study is concerned with efforts to limit the hazards involved in preparing viral antigens. A recent study by LoGrippo and Hartman(7) has shown that Beta-propiolactone (BPL) promptly inactivates certain viruses without seriously reducing their ability to elicit neu-

* This work was supported by contract with Chemical Corps, Fort Detrick, Frederick, Md.

tralizing and protective antibodies as compared with living vaccines and formalin- and phenol-inactivated vaccines. Similarly, Mack and Chotisen(8) have demonstrated that BPL-treated Newcastle Disease virus is noninfectious for chickens but induces substantial neutralizing and hemagglutination-inhibition antibody formation. A high potency BPL-killed fixed rabies virus vaccine has been reported by Powell and Culbertson(9). *BPL* shows promise for use in sterilizing arterial homografts(10,11,12) and perhaps, coupled with UV irradiation, in sterilization of serum for transfusion(13). These reports suggested to us a possible application of this reagent in rendering viral preparations safe during processing of antigens for use in *in vitro* tests.

Materials and methods. Complement-fixing antigens were prepared from brains of 3-day-old mice inoculated intracerebrally with 0.03 ml of 10^{-4} dilution of SLE virus (Hubbard strain) infected mouse brain according to modification of technic of Havens *et al.* (14). The infant mice were sacrificed by exsanguination after onset of symptoms and their brains removed and frozen at –40°C. Twenty % suspensions were made by thoroughly grinding infected brain tissue in the cold with fine glass beads, after which saline and sufficient M/1 Na_2HPO_4 were added to produce final pH of about 8.0. In the case of antigens treated with Beta-propiolactone,† sufficient BPL from freshly prepared 10% stock solution was incorporated in the saline to give a final concentration of 0.2%. Preparations of normal infant mouse brains were similarly treated for complement-fixation test controls. Infected brain suspensions, BPL-treated and control, were stored 2 hours at 4°C or 37°C when aliquots were removed for infectivity titrations in 12 to 15 g mice. The suspensions were centrifuged in the cold at 3000 rpm for 15 minutes to remove gross sediment and then the supernates were centrifuged at 13,000 rpm in Spinco centrifuge for 1 hour. Merthiolate (1:10,000) was added to supernates which were stored at 4°C in screw cap tubes. The hyperimmune sera were prepared by immunization of guinea pigs with hamster brain infected with St. Louis Encephalitis (SLE), Murray Valley Encephalitis (MVE), Japanese Encephalitis (JE), and West Nile (WN) viruses as previously described(15). The SLE antiserum used for the investigation of the reactivity and stability of antigen preparations fixed complement at a dilution of 1:64 when tested against a commercial SLE CF antigen (Lederle) and showed no fixation with normal mouse brain antigen. All sera were inactivated at 56°C for one-half hour prior to use in complement-fixation tests. Paired human sera from patients with St. Louis Encephalitis were received from the following persons to whom we are indebted: SLE sera nos. 1447, 1448, 1449, 1469, 1503 and 1504 were provided by Dr. S. S. Kalter, CDC, Montgomery, Ala.; nos. 1, 2, and 3 and horse Western Equine Encephalitis (WEE) serum from Dr. J. V. Irons, Texas Department of Health, Austin; and sera from patients with Eastern Equine Encephalitis (EEE) from Dr. Roy F. Feemster and Mrs. J. B. Daniels, Mass. Dept. of Public Health, Boston. The technic for the complement-fixation test was modified from Casals(16). Complement titrations were performed in the presence of dilutions of each antigen. The final volume of reactants was 1.5 ml including 0.25 ml each of appropriate antigen and serum dilutions, 0.5 ml of guinea pig complement (Sharp and Dohme) diluted to contain 2 exact units, and equal volumes of rabbit anti-sheep erythrocyte hemolysin (3 units) and 3% sheep erythrocytes in 0.5 ml. The antigens, serum and complement were incubated overnight at 4°C before addition of the hemolytic system. Tests were then incubated for 30 minutes at 37°C and read visually. In some cases, owing to a scarcity of serum, CF tests were performed with half the above volumes of reactants.

Results. The LD_{50} titer of SLE stock suspensions prepared in the described manner was around 7.22 calculated by the method of Reed and Muench(17). After BPL treatment, either at 4°C or 37°C for 2 hours, no infectious virus could be recovered in undi-

† BPL was obtained through the courtesy of T. L. Gresham, B. F. Goodrich Co., Cleveland, O.

TABLE I. Complement-Fixation with BPL-Treated and Control SLE Antigens.

Hyperimmune serum		CF titer* SLE (1:8)	CF titer* BPL-SLE (1:8)
Guinea pig	SLE	128	128
	MVE	16	16
	WN	32	32
	JE	8	8
	Normal	<4	<4
Horse	WEE	"	"
Human (conv.)	EEE	"	"

* Reciprocal of highest serum dilution showing 50% fixation.

luted stock suspensions or serial dilutions thereof by intracerebral inoculation of 12 to 15 g mice.

The reactivity of BPL-treated antigens with SLE antiserum was essentially the same as control preparations. CF titers as high as 1:256 were achieved with BPL-treated and untreated preparations although a one tube loss in titer was occasionally encountered with BPL treatment. Since BPL hydrolyzes to yield acidic products, care must be taken to maintain the pH by the addition of M/1 Na_2HPO_4. The antigens are relatively stable on storage at 4°C for at least 3 months. In addition, BPL-treated and control antigens were lyophilized and reconstituted after one month storage at 4°C with only a one tube drop in titer in each instance.

In the data presented in Table I, it can be seen that the degree of cross reactivity of the BPL-treated SLE antigen with sera of the JE-WN-SLE-MVE group is the same as the untreated antigen and similar to that reported in a previous study(15) in which benzene extracted antigens were used. In addition, tests with paired human patients' sera (Table II) demonstrated that the BPL-treated antigen was equally effective in detecting complement-fixing antibodies as its infectious counterpart and seems to be equally as sensitive as the antigens used in the laboratories from which the sera were obtained.

Summary. A method is described for preparing a complement-fixing antigen of SLE virus which is non-infective for mice. The antigen is treated in an early stage in its preparation with Beta-propiolactone, a potent virucidal agent. The liquid antigen is relatively stable on storage at 4°C and may be lyophilized without significant loss of reactivity. The BPL-treated antigen exhibits the same degree of cross-reaction with other members of the encephalitis group as untreated preparations and previously described antigens. Tests with paired patients' sera have indicated its usefulness as a diagnostic aid. The use of BPL as a virucidal agent in the preparation of non-infectious inactivated viral antigens should constitute a distinct advantage over previously described technics.

TABLE II. Complement-Fixation Tests with BPL-Treated SLE Antigen and Human Immune Sera.

Patient serum No.	Time after onset		CF titer SLE	CF titer BPL-SLE
1447	Acute	(8)*	8	8
	Conv.	(8)	16	16
1448	Acute	(2)	<4	<4
	Conv.	(8)	16	16
1449	Acute	(<2)	8	4
	Conv.	(8)	32	32
1469	Conv.	(16)	32	32
	Post conv.	(4)	16	16
1503	Acute	(2)	16	16
	Conv.	(32)	64	64
1504	Acute	(<2)	<4	<4
	Conv.	(64)	128	128
1	Acute	(±4)	<4	<4
	Conv.	(32)	16	16
2	Acute	(4)	8	8
	Conv.	(32)	32	32
3	Acute	(<4)	<4	<4
	Conv.	(128)	64	64

* Complement-fixing titer obtained by laboratory donating the serum.

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Studies in Marine Biology. II. *In vitro* Culture of Zooxanthellae.* (23139)

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In November, 1956, the authors examined a wide variety of marine invertebrates from the waters around Bimini, B.W.I. We were interested in the algal cells—the so-called symbiotic zooxanthellae—which live within the tissues of many radiolarians, coelenterates, molluscs, bryozoa, worms, ascidians, etc. (1,2). With a few exceptions, firm taxonomic placement of zooxanthellae has been difficult because of their somewhat generalized morphology and because of absence or elusive nature of a motile phase(3,4); only Kawaguti(5) has reported seeing motile forms. Furthermore, whether zooxanthellae of various host animals fall into one or into multiple taxonomic groups has not been established. As to their role as symbionts, one school(6,7) holds that the zooxanthellae do not appreciably contribute to nutrition of the host; another, based largely on recent work of Sargent and Austin(8) and of Odum and Odum (9), suggests that the abundant presence in coral reef communities of photosynthetic algae (including both interstitial zooxanthellae and intraskeletal filamentous algae) may well be crucial to growth and maintenance of the entire reef biotope.

If, following on the work of Kawaguti, zooxanthellae could be isolated from the host animal, grown serially in bacteria-free culture, and their nutritive and reproductive characteristics studied, not only should their identity in each case be ascertainable, but also possibly clues as to their trophic relationship with the host animal. The aim of the present study was to isolate certain coelenterate zooxanthellae and to culture them *in vitro*.

Materials and methods. A large zooxanthellae-containing Scyphozoan (*Cassiopeia* sp.) and a large zooxanthellae-containing anemone (*Condylactis* sp.) were selected. *Cassiopeia* abounded in waters around S. Bimini, B.W.I. *Condylactis* was similarly abundant at E. Bimini. The brown-green coloration of both these animals reflects the presence, mainly in endodermal tissues, of densely packed layers of zooxanthellae (Plate I, Fig. 1 and 2). To secure zooxanthellae free from host tissue for *in vitro* culture, an entire live specimen of either *Cassiopeia* or *Condylactis* was washed successively in tapwater and sterile seawater, then macerated in a Waring blendor with approximately 100 ml of sterile seawater. Within a few minutes tissues were

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reduced to a soupy fluid, which was immediately filtered through glass wool. The filtrate was centrifuged at moderate speed until a sediment, consisting of packed algal cells largely freed from host tissue, collected on the bottom of the tubes. The supernatant was discarded, and the sediment resuspended in sterile seawater. Centrifugation and resuspension were repeated 6 times, with the last 3 washings made in synthetic seawater media. Finally, the sediment was resuspended in media containing different concentrations of

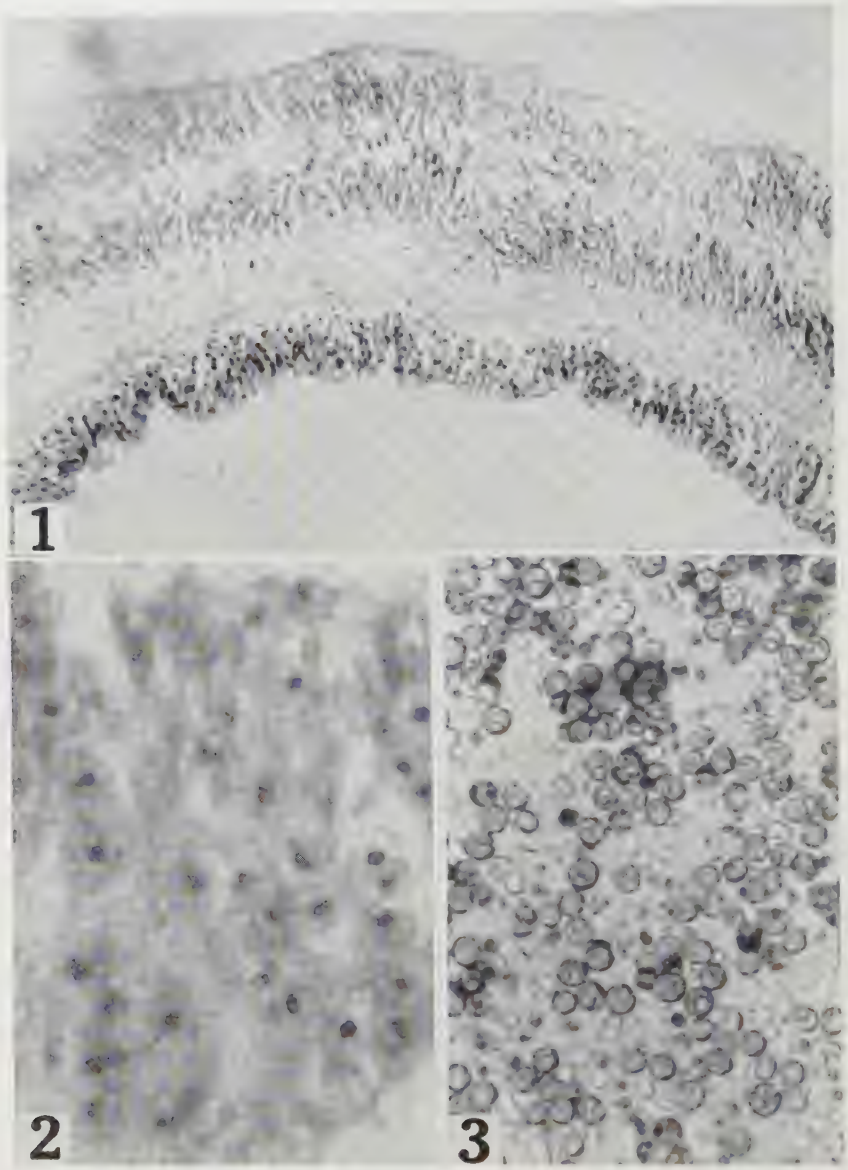

Plate I.

FIG. 1. Cross-section of portion of anemone *(Condylactis)* tentacle to show endodermal location of zooxanthellae cells. Bouin's, H & E. Mag. × 140.

FIG. 2. Detail of endoderm of anemone tentacle to show interstitial position of zooxanthellae cells. Bouin's, H & E. Mag. × 1000.

FIG. 3. Living vegetative zooxanthellae cells removed from anemone tissue by methods described in text. Mag. × 400.

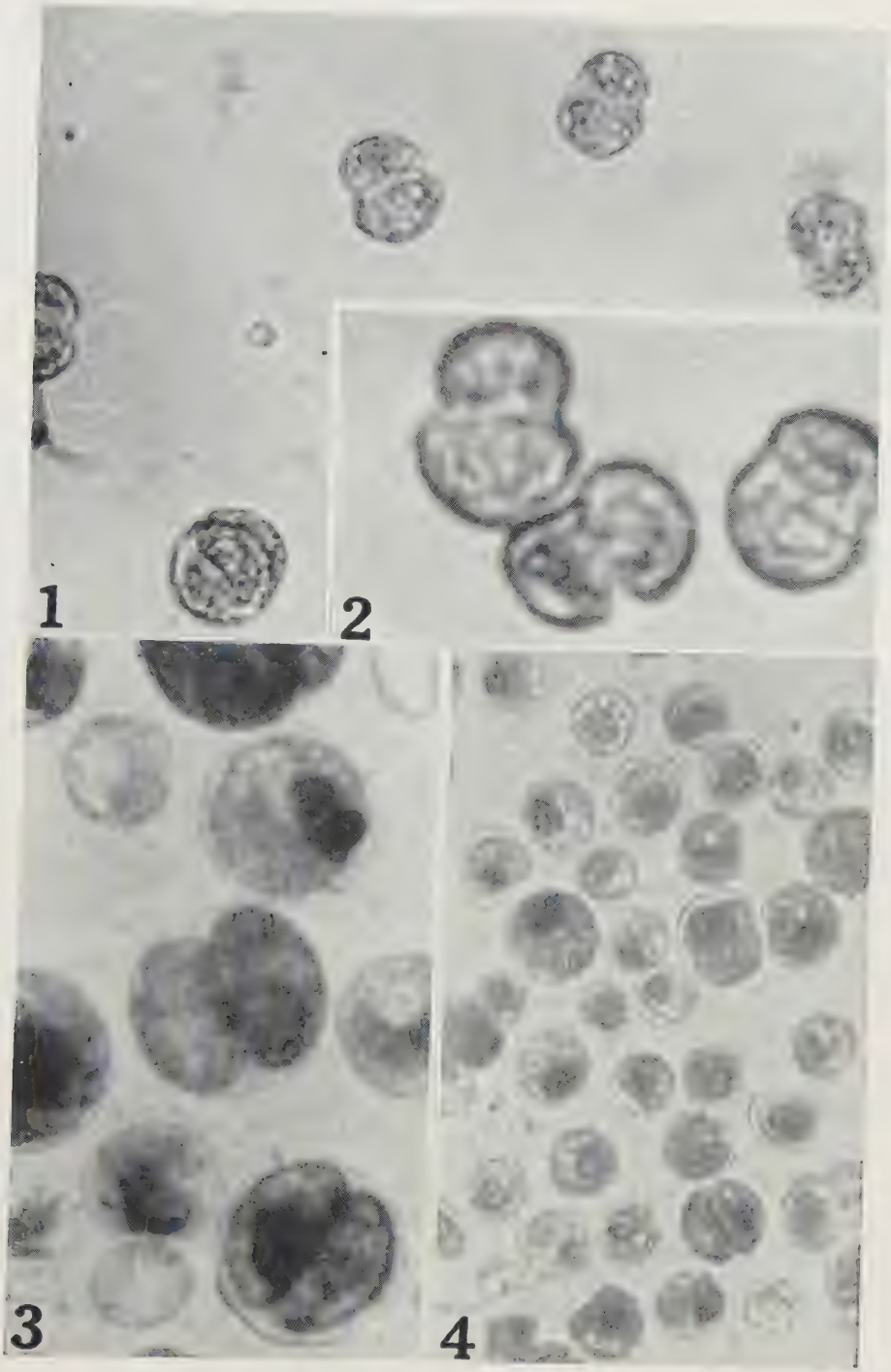

Plate II.

FIG. 1. Living motile cells derived *in vitro* from *Cassiopeia* zooxanthellae. Flagella not visible. Cell in lower part of picture had reverted from motile to spherical non-motile form only a few seconds before photograph was made. Mag. × 3750.

FIG. 2. Enlargement of 3 living motile cells of type shown in Fig. 1 (above). Mag. × 7500.

FIG. 3 & 4. Living vegetative zooxanthellae (from *Cassiopeia*) to show various division stages. Mags. × 2100 and × 1000.

an antibiotic mixture (1 ml of mixture contained: penicillin G, 46,000 units; dihydrostreptomycin, 2,000 μg; chloromycetin, 5,300 μg; neomycin, 12,000 μg) sterilized through a Seitz filter. The most suitable non-toxic concentration of the antibiotic mixture in the wash medium was 5 ml %. Centrifugation from and resuspension in such antibiotic-containing media was repeated 3 times; the cells were allowed to stand in the last wash 1½ hours. A wet-slide preparation of this suspension revealed algal cells in dense concentration. Some showed division stages (Plate II, Fig. 3 and 4); there were no motile organisms. These vegetative cells, 7-14 μ diameter, contained chromatophores, a nucleus, and various vacuoles and droplets. A remarkable feature of these vegetative cells is the toughness of their outer membrane, as indicated by the lack of distortion following centrifugation. The membrane's osmotic character is indicated by a tolerance of high antibiotic and nutrient concentrations, as well as of washings in freshwater. Further, cells on dry agar for as long as 15 days were viable when transferred to liquid media.

The next step was enrichment of seawater and synthetic media. Two different basal marine media, both known to support growth *in vitro* of a number of marine microorganisms (10), were selected as starting points.† In empirical concentrations ranging from 1 to 100 mg %, one, some, or all of the following supplements were added to further enrich the basal media: milk protein hydrolysate, serum extract, corpus luteum extract, yeast autolysate, Trypticase, brain-heart extract, desiccated bone marrow, and sterile Seitz-filtered juice from the macerated tissues of *Cassiopeia* or *Condylactis*. Our selection of organic supplements and variations of inorganic concentrations was based on earlier microbiological work at the Haskins Laboratories. By adding these supplements it was hoped to supply nutritional sources and physiological conditions necessary for proliferation of zooxanthellae *in vitro*. Such enriched media were made up in liquid, solid (1.5% agar,) and semi-solid (0.5% agar) form. For suppression of bacterial growth, all media received at least 1 ml % of the antibiotic mix.

Hundreds of media permutations were tested. There is no need to list all, for while good growth was observed in a number of the media, the combination that seemed to encourage proliferation most profusely and most consistently, is: *Basal Medium B,*† to which was added 1 ml % of amino acid mix,‡ 0.01 mg % yeast autolysate; 1.5% agar: at 24-26°C. Ten ml of agarized medium was slanted in screw-cap test tubes (20 X 125 mm), and 2-4 ml of liquid medium plus antibiotic were added to the slanted surface prior to inoculation with 1-3 drops (5-10,000 cells) of zooxanthellae suspension. The physical environment of the inoculated cells was thus biphasic (solid-liquid). An additional type of inoculum consisted of fragments of zooxanthellae-containing tissue cut directly from the living *Cassiopeia* or *Condylactis*. Such fragments, before being added to the nutrient tubes, were washed repeatedly in sterile seawater, then in sterile media-plus-antibiotics solution, as described above. The tissue fragments were allowed to stand in the last antibiotic wash for 1½ hours, then were washed repeatedly in sterile media, and transferred to the final culture plate or tube. The zooxanthellae of such tissue fragments showed growth characteristics and media preferences similar to those of zooxanthellae introduced as suspensions.

† A. *Enriched Seawater Medium* (ASW-III), containing: aged seawater, 100 ml; KNO_3, 20 mg; K_2HPO_4, 2 mg; Fe (as Cl), .01 mg; soil extract, 4 ml; Mn (as Cl), .04 mg; Na-H glutamate, 50 mg; glycine, 50 mg; vit. mix No. 8, .1 ml; liver OXOID, 1 mg; pH 7.4-7.6. B. *Artificial Seawater Enriched Medium,* containing: NaCl, 2.8%; KCl, .06%; $MgSO_4 \cdot 7H_2O$, 0.6%; Fe (as Cl), 0.13 mg%; Zn (as Cl), 15 μg%; Mn (as Cl), 0.12 mg%; Co (as Cl), 0.3 μg%; Cu (as Cl), 0.12 μg%; Na_2EDTA, 3 mg%; Na_2CO_3, 5 mg%; Ca (as Cl), 15 mg%; NTA, 10 mg%; $NaNO_3$, 1 mg%; K_2HPO_4, 0.1 mg %; vit. mix No. 8, .05 ml%; B_{12}, .01 μg%; thiamine HCl, .05%; Tris buffer, .05%; pH 8-8.3. For formulation details of these basal media, see (10).

‡ 1 ml of amino acid mix contained: Na-H glutamate, 10 mg%; DL-alanine, 10 mg%; DL-aspartic acid, 10 mg%; DL-asparagine, 10 mg%; DL-methionine, 1 mg%; L-histidine HCl, 1 mg%.

Results. After inoculation, some tubes and plates were kept in the dark, some in continuous light (5-7 inches from 40-watt, cool, white, fluorescent source), others in alternating light and dark (8-8, 12-12, and 24-24 hours), at 24-32°C. In some tubes, motile cells began to appear within 3 days, especially if subjected to alternate light and dark at 12-hour intervals. These motile forms (4-6 μ diameter) were unequivocally identified as dinoflagellates, both for the *Cassiopeia* and the *Condylactis* zooxanthellae (Plate II, Fig. 1 and 2). They showed: (*a*) a transverse girdle; (*b*) 2 flagella, one enclosed in the girdle and the other extending backwards; (*c*) a characteristic spiral motility. Identification of these zooxanthellae as dinoflagellates supports earlier conclusions of Hovasse(3) and Pringsheim(4) for corals based tentatively on the morphology of resting cells, and of Kawaguti(5) based on observation of motile cells. Whether zooxanthellae vary according to the host species, the season of the year, or local ecological conditions, is presently unknown.

Our motile cells stayed in this condition for a few minutes to 6 hours. Increased light intensity regularly shortened the motility period. In losing motility, the cells cast off their flagella, became nearly spherical, and assumed a quiescent form resembling the vegetative cells (Plate II, Fig. 1, lower specimen). That these motile cells were actually being derived from the original vegetative form of the zooxanthellae of the inoculum type, and not from any contaminant organisms, was indicated by the nature of the reversion and by the observation of motile forms breaking out of dividing cells within the vegetative population. Further, vegetative cells (from 15-day-old dry agar plates which in daily microscopic observation showed no motile cells) when introduced to liquid medium, produced a profusion of flagellated cells within 1-3 days. Our original cultures of zooxanthellae have now undergone eight serial passages with no loss of integrity or virulence; inocula of 5-10,000 cells show an absolute increment within 15 days to about 30 million. Serial transfers are now routine in our laboratory. Single-cell transfers have not been attempted, pending completion of further nutritional studies to determine optimal growth conditions.

Proliferation of the zooxanthellae *in vitro* appears to be of 3 types: (*a*) vegetative cells giving rise to vegetative cells; (*b*) vegetative cells giving rise to motile cells; and (*c*) motile cells reverting to the vegetative form (Plate II, Fig. 1, 3, 4). The mechanics of such transitions awaits further study.

Motile forms were observed only in liquid or semi-solid media. No motile forms were ever observed in the coelenterate tissue either before or after maceration, or in samples of enteric or tentacle fluid taken by hypodermic puncture.

What factors control transition from the nonmotile to motile state of these organisms are not yet clear; light is certainly a factor, as is, of course, the inorganic and organic makeup of the nutrient medium. Further analysis of growth requirements continues.

Summary. 1. Single-cell algae (the so-called symbiotic zooxanthellae) living interstitially in a marine jellyfish and a sea anemone were isolated from the host tissue and cultured *in vitro*. 2. In culture the zooxanthellae gave rise to motile forms whose morphology clearly marked them as dinoflagellates. 3. Isolation procedures and some nutrient requirements for the *in vitro* culture of such zooxanthellae are described, as well as methods used for serial passage.

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Cultivation of Measles Virus in Human Amnion Cells and in Developing Chick Embryo.* (23140)

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Enders and Peebles(1) presented evidence indicating that the virus of measles can be isolated in cultures of human and monkey renal epithelial cells from the blood and throat washings of patients in the early stage of the disease. Capable of continuous multiplication in such cultures, the agent produces a characteristic cytopathogenic effect which is specifically prevented by sera of man or monkey taken during the convalescent phase of measles. Moreover, an antigen appears in the fluid of infected tissue cultures that fixes complement specifically in the presence of convalescent phase measles sera(1,2,3). These findings have made it possible to investigate more conveniently and precisely the behavior of the virus in other systems. In this communication we shall describe the propagation of the virus and the cytopathic changes it induces in cultures of human amnion cells. In addition we shall record details of the procedures by which evidence of its multiplication in chick embryos was obtained. A summary account of these experiments has recently been published(2).

Methods and materials. Viruses. The "Edmonston" strain(1) of measles virus was most extensively employed. This agent was isolated in February, 1954, from the blood of a typical case of measles on the first day of the rash. From that time until January, 1955, it was subjected to a total of 24 passages in roller tube or trypsinized monolayer cultures of human postnatal renal cells maintained in bovine amniotic fluid medium. To a less extent certain other strains isolated in this laboratory were studied in respect to their capacity to multiply in human amnion cells and in chick embryos.

Tissue cultures. Suspensions of amnion cells were prepared according to the method of Zitcer and her coworkers(4). Approximately 300,000-400,000 cells in a volume of 1 ml of medium were allowed to settle on the wall of a tube 15 x 150 mm held at an angle of about 5° from the horizontal. During the period of cell outgrowth the medium consisted of the following constituents:

Normal horse serum (inactivated)	20	%
Bovine embryonic extract	5	%
Bovine amniotic fluid	37.5	%
Hanks' balanced salt solution	37.5	%
Streptomycin	100	mg/ml
Penicillin	100	units/ml
Mycostatin	100	"

In the most recent experiments the following medium, which gives equally satisfactory results, has been used to promote cell outgrowth:

	%
Normal horse serum (inactivated)	20
Hanks' balanced salt solution	70
L-Glutamine (2.9 mg/ml H_2O)	10
Antibiotics in quantities indicated in formula above.	

When the monolayer was established and at the time the virus was introduced the medium used to promote cell outgrowth was replaced

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by a mixture of the following composition:

	%
Normal horse serum (inactivated)	5
Bovine embryonic extract	5
" amniotic fluid	45
Hanks' balanced salt solution	45

Antibiotics as given in first formula.

Suspensions of trypsinized human renal cells were prepared according to the procedure described by Younger(5) for monkey renal cells. The renal tissue was obtained at operations for reduction of hydrocephalus by ventriculo-ureteral shunt. The 5% normal horse serum-bovine amniotic fluid medium was employed throughout in the cultivation of these cells. *Embryonated eggs.* Technical details are included in the description of experiments on cultivation of the virus in chick embryos.

Results. Cultivation of measles virus in *human amnion cells.* Undiluted tissue culture fluid of the 24th human renal cell passage of the Edmonston strain was used as inoculum to initiate a series of 28 passages in monolayer cultures of human amnion cells. Volume of inoculum was 0.1 ml which contained $10^{2.8}$ TCD_{50} virus as determined in cultures of human renal cells.

Some difficulty was experienced in adapting the Edmonston virus to growth in the amnion cells. During earlier passages the cytopathic changes extended very slowly throughout the monolayer and, as compared with those seen in cultures of human renal cells, appeared after a longer interval, *i.e.* 3 to 4 days later. In attempts to adapt to human amnion cells 6 additional strains of measles virus that had been cultivated for a varying number of passages in human renal cells, inocula of 0.2-0.4 ml in 4 cases resulted in proliferation of virus when 0.1 ml proved ineffective. In the original amnion cell cultures of these strains, cytopathic changes were again slow to appear and in subsequent passages viral multiplication in certain instances did not occur.

Cytopathic changes in amnion cells. The changes induced by the Edmonston strain in human amnion cells are of 2 distinct types. The first consists in the syncytium-like or multinuclear giant cell formation which characteristically reflects the multiplication of the virus in cultures of human or monkey kidney cells(1,2). The syncytia are composed of a broad expanse of cytoplasm that includes numerous nuclei. Eosinophilic intranuclear inclusions are a prominent feature in this "lesion." Irregular masses of strongly eosinophilic amorphous material surrounded by a clear zone are often seen in the cytoplasm. Although doubtful at first respecting the significance of these cytoplasmic bodies, we have recently become convinced that they also are specific manifestations of cytopathogenicity, since we have never observed them in uninoculated cultures. The appearance of the syncytial change in an early amnion cell passage is shown in Fig. 1. The second type of change, (Fig. 2), consists in the assumption by an increasing number of epithelial cells of a spindle-like or, in certain instances, stellate configuration. Concomitantly refractility of the affected cells increases, rendering them conspicuous amid the more translucent normal cells. Margins of the elongated cytoplasmic processes distinguishing the affected cells are somewhat irregular or shaggy and occasionally exhibit a fine "beading." Usually these changes are first seen in a few cells lying adjacent to each other. Later much of the cell population becomes involved and the process slowly terminates in complete cellular disintegration. As passage of the virus in successive cultures of amnion cells was continued, the transformation of spindle cells

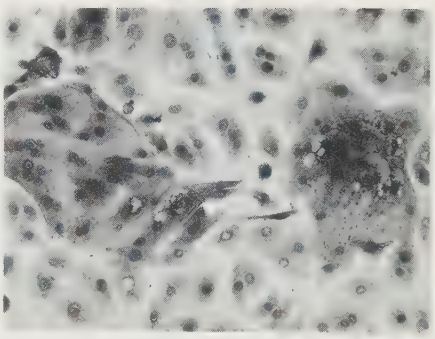

FIG. 1. Cytopathic changes in human amnion cells 16 days after inoculation of Edmonston measles virus of the 24th passage in human renal cells. Note syncytia or "multinuclear giant cells" and absence of spindle forms. Bouin's fixative. H & E stain. Mag. 107 ×.

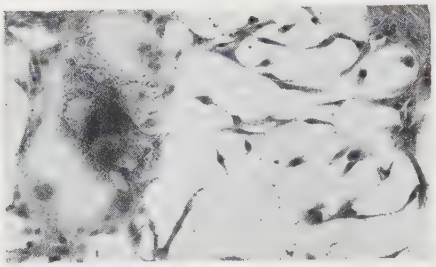

FIG. 2. Changes in human amnion cells 16 days after inoculation of Edmonston virus from 24th passage in these cells. Note spindle forms together with a syncytium in which irregular cytoplasmic inclusions are visible. Bouin's fixative. H & E stain. Mag. 85 ×.

tended to predominate over formation of syncytia. Indeed, in many cultures, particularly when small quantities of virus were inoculated, only spindle cells were seen. Examination of stained material revealed eosinophilic intranuclear inclusion bodies in certain of the spindle cells that closely resembled those occurring in the nuclei of the syncytia. The spindle cell change was first definitely associated with viral activity when in cultures of the 14th passage it was seen to involve a large proportion of the cell population. We now know from results obtained by reexamining the cytopathogenic effects of virus from certain of the earlier amnion cell passages that the Edmonston strain was capable of inducing this type of change at least by the fourth passage. Fluids from earlier passages were not available for test. This change, however, has not been observed in the original human amnion cell passages of 6 other strains of virus. Whether it will appear as serial passage of these agents is continued remains to be determined.

Spindle cell formation an attribute of measles virus. To show that the spindle cell transformation represents another expression of cytopathogenicity of the measles virus, neutralization and complement fixation tests were carried out with acute and convalescent phase human and monkey sera. The results are included in the first 2 rows of Table I. In these experiments tissue culture fluid of the 25th and 26th renal cell passages (designated "renal cell" virus) and the 28th amnion

TABLE I. Neutralization and CF Tests with Edmonston Virus Grown in Human Kidney and Amnion Cells and in Chick Embryos.

	Human sera*				Monkey sera†			
	Neut. test		CF test		Neut. test		CF test	
Source of virus	Acute	Conv.	Acute	Conv.	Acute	Conv.	Acute	Conv.
Human kidney P25 + P26‡	<8	177¶	<4	128**	<4	175	<4	32
Human amnion P28§	"	355	"	128	"	609	"	128
Egg P 1‖	—††	—	—	—	—	—	"	256
2	—	—	—	—	<4	2430	"	"
3	—	—	—	—	—	—	"	128
4	<8	1200	—	—	—	—	—	—
9	<16	609	—	—	<16	180	"	16

* Sera of patient from whom Edmonston strain was originally isolated. Acute phase specimen taken on 1st day of rash; convalescent 58 days later.

† Acute and convalescent sera from 3 monkeys were used: CF and neut. tests with virus from human kidney, human amnion and Egg P 9 were done with sera from Monkey 69 inoculated with 23rd human kidney passage Edmonston strain(6); sera from Monkey 78 inoculated with the same virus were used in neut. tests with Egg P 2; sera from Monkey 52 inoculated with the 1st passage in human kidney cells of the Edmonston strain(6) were used in CF tests with Egg P 1,2,3.

‡ Fluids from 25th and 26th passages in human kidney cells were respectively used in neut. and CF tests.

§ Virus from 28th passage in human amnion cells.

‖ Egg P 1 = first egg passage. Infected chick amniotic membrane suspensions were used in neutralization tests with egg-propagated virus. Pooled TC fluids from cultures of human amnion cells inoculated with virus from chick embryo passages were used as antigens in CF tests.

¶ Reciprocal of neutralizing titer of serum against 100 TCD_{50} calculated by formula of Reed and Muench.

** Reciprocal of highest dilution of serum fixing complement with 2 units of antigen. Tests carried out by technic of Fulton and Dumbell(7) as modified by Svedmyr and co-workers(8).

†† Not done.

TABLE II. Infectivity and Cytopathogenic Effect of Edmonston Virus in Cultures of Two Kinds of Human Cells.

Source of virus	Culture: Human kidney* CPE‡	Human kidney* Infect§	Human amnion† CPE	Human amnion† Infect
Hu am P28‖	Syncyt¶	3.5	Sp cells**	3.5
Egg P6††	"	3.8	"	3.8

* Trypsinized human kidney cells.
† " " amnion " .
‡ Cytopathogenic effect.
§ Infectivity titer: log TCD_{50}/0.1 ml.
‖ Fluid from 28th passage in human amnion cells.
¶ Syncytial formation.
** Predominantly spindle cell formation.
†† Pooled chick amniotic membrane suspension of 6th egg passage.

cell passage (designated "amnion cell" virus) were employed as antigens. The "renal cell" virus induced only formation of syncytia in cultures of human kidney cells whereas the effect of the "amnion cell" virus consisted predominantly or solely in development of spindle forms. Cultures of human renal cells were used in the neutralization tests with "renal cell" virus and cultures of human amnion cells in the tests with "amnion virus." One of the 2 pairs of sera was obtained from the patient yielding the Edmonston virus; the other from a cynomologus monkey that exhibited typical signs of measles following inoculation of fluid from the 23rd passage of this agent in human renal cells(6).

The data in Table I indicate that the acute phase sera in the lowest dilutions tested failed to prevent the cytopathic changes induced by either "renal cell" or "amnion cell" virus. In contrast the convalescent phase sera in high dilution proved inhibitory. Similarly, these sera brought about fixation of complement in comparable dilutions in the presence of both antigens while the active phase sera did not. Because these findings show that the virus capable of inducing the spindle cell change in amnion cells is antigenically similar or identical with the agent propagated only in human renal cells, it may be concluded that the measles agent itself is responsible for this newly recognized cytopathic effect.

Additional data supporting this conclusion were obtained in titrations of virus of the 28th amnion cell passage carried out simultaneously in cultures of human amnion and kidney cells. The results are summarized in the upper row of Table II. In both systems the endpoint of viral infectivity proved to be the same. In the renal cell cultures, however, only syncytia were observed, whereas only spindle cells were seen in the amnion cell series.

Cultivation of measles virus in the developing chick embryo. Nearly 2 decades ago several workers reported the successful propagation of measles virus in the developing chick embryo(9). Of these reports the most convincing were those of Rake and Shaffer(10, 11). Similar attempts by others, however, yielded inconclusive or negative results. This situation in retrospect is, perhaps, not surprising since at that time the only means of demonstrating the presence of the virus was by inoculation of monkeys and it has recently been demonstrated(6) that many normal monkeys possess naturally acquired immunity to the agent. Because of the conflicting results of these earlier workers, susceptibility of the chick embryo to infection with measles virus has remained uncertain, although in 2 recent communications, one from Japan(12) and the other from Russia(13), further evidence is presented suggesting that this agent may be propagated in this system.

When it was found that multiplication of measles virus could be accurately and conveniently demonstrated and measured in tissue culture, Enders and Peebles(1) attempted to repeat the experiments of Rake and Shaffer(11), using the tissue culture technic to determine presence or absence of the agent in various constituents of the chick embryo. Following the procedures described by Rake and Shaffer for establishment and maintenance of the virus in the developing hen's egg, no evidence of viral multiplication was obtained. Subsequently additional experiments with 5 strains of measles virus were carried out in this laboratory in which the same technics were employed. However, even in materials taken from the original egg passages, no virus was demonstrated.

Recognition of the capacity of the Edmonston strain to induce the spindle cell change

after many passages in cultures of 2 different kinds of human cells suggested that this newly observed cytopathic effect might be the result of a mutation which in turn might be accompanied by a change in the capacity of the virus to multiply in the chick embryo. Although results subsequently obtained have not supported this hypothesis, a series of egg passages was then initiated using as the original inoculum virus that had been cultivated during many passages in amnion cells.

Passage of virus in chick embryos. We have emphasized the fact that the technic employed in our unsuccessful attempts to cultivate the virus in this host was essentially that of Rake and Shaffer(11). Their usual procedure consisted in inoculation of the chorioallantois and the harvesting of materials for passage after an interval of 4 to 7 days. In the experiments to be described in which viral multiplication was finally demonstrated, the inoculum was introduced into the amniotic sac and the embryonic materials were collected as routine 9 days thereafter. These modifications were prompted by 2 considerations. The amniotic route was selected, since it seemed possible that the virus, now adapted to human amnion cells, might more readily proliferate in the analogous cells of the chick. The eggs were incubated for a longer period because in an experiment with virus from an earlier amnion cell passage the agent was recovered 9 days after inoculation of the embryos. Although at that time it was not demonstrated in subsequent egg passages, this finding suggested the possibility that slight multiplication may have occurred in the first passage after a period of incubation longer than that previously allowed.

In Table III are summarized details of procedure and the results obtained during 12 serial passages of the Edmonston virus in chick embryos. The original inoculum consisted of tissue culture fluid from the 28th human amnion cell passage. Chick amniotic or pooled amniotic and allantoic fluid was used as the inoculum in the first 6 sub-passages. Thereafter a pool of amniotic fluid and membrane suspension was employed, since by that time it had been determined that the amniotic membrane consistently contained the largest amount of virus. The em-

TABLE III. Serial Passages of Measles Virus: Edmonston Strain in Chick Embryos.

		Titer material harvested†				
		Amnion		Chorioallantois		Embryo
Passage	Inoculum*	Fluid	Tissue	Fluid	Tissue	
I	TCF hu am P28‡	+	NT§	—	NT	NT
II	Pooled fls	2.5‖	NT	2.5‖	NT	NT
III	*Idem*	.8	2.8¶	—	2.8¶	—
IV	Am fl	.5	3.5	.3	2.3	1.0
V	*Idem*	1.3	3.5	—	+	+
VI	"	.5	2.8	—	—	+
VII	"	—	1.5	—	—	—
VIII	Pool am fl and membr	—	3.5	—	—	—
IX	*Idem*	.8	3.5	+	+	+
X	"	1.8	2.8	+	+	+
XI	"	2.3	4.3	—	+	+
XII	"	2.3	4.5	NT	+	+

* Introduced into amniotic sac under direct vision. 5 embryos were inoculated with 0.2 ml of undiluted materials from previous passage except as noted.

† Infectivity titers expressed as log ID_{50}/0.1 ml. In certain instances where titrations were not done presence of virus in the material is indicated by "+"; failure to detect its presence by "—." Aliquots of 0.1 ml of undiluted materials or dilutions thereof were added to each of 3 cultures of human amnion cells. Based on an estimation of the wet volume, 10% suspensions of chick tissues were prepared in tissue culture medium for use in titrations or tests for presence of virus.

‡ Titer tissue culture fluid used as original inoculum = 4.0/0.1 ml; vol inoculated = 0.5 ml.

§ Not tested.

‖ Each of 2 sets of 5 embryos inoculated with pooled am and al fluids.

¶ Am and C-A membranes pooled and titrated. Collateral passage line initiated from 1 embryo dying on 8th day.

bryos were incubated for 6 days at about 38°C before the inoculum was introduced and at 36°C thereafter. The presence of virus in various embryonic constitutents was determined by adding the latter to cultures of human amnion cells. This procedure was necessary because no definite changes indicative of viral multiplication were observed within the contents of the egg itself. So far attempts have also failed to demonstrate the presence of viral complement fixing antigen and hemagglutinin in egg materials shown to contain the virus. Inspection of the titrational data (Table III) for the materials derived from successive passages leaves no doubt that viral multiplication occurred. Thus it may be conservatively estimated that the original inoculum by the 12th passage was diluted 10^{12} x taking the volume of fluid in the amniotic sac as 2 ml. This dilution factor exceeds by about 10^{7} x the number of infectious doses of virus introduced as inoculum in the first passage. Furthermore, beginning with the 4th passage the quantity of virus found in the amniotic membranes in most cases definitely exceeded that added as inoculum. With amniotic fluid of an embryo dying on the 8th day in the 3rd passage (see Table III), a collateral series of 8 egg passages was initiated. The amount of virus found in the various materials of each passage was in general comparable to those recorded in Table III. Similarly, during the course of 3 successive egg passages of another collateral line established with virus from amniotic membranes of the surviving embryos of the 3rd passage of the original line, evidence of multiplication was obtained in each passage.

Identification of virus. The agent propagated in the chick embryo was identified as measles virus in neutralization and complement fixation tests in which acute and convalescent phase human and monkey measles sera were employed. From the data recorded in the lower part of Table I it is apparent that titers of the convalescent sera in the presence of virus derived from various egg passages were of the same order of magnitude as those found for measles virus which had been maintained continuously in cultures of human renal and amnion cells. The identity of the

FIG. 3. Changes induced in human amnion cells by Edmonston virus of 3rd chick embryo passage 14 days after inoculation. Note predominance of spindle forms. A small giant cell is also visible, *Cf.* Fig. 2. Bouin's fixative. H & E stain. Mag. 230 ×.

agent cultivated in chick embryos was also supported by the fact that its cytopathogenic effect on human amnion cells, which consisted predominantly in spindle cell transformation, (Fig. 3), and on human kidney cells in which only syncytial changes were noted (Table II) was comparable to that of the virus used to initiate the series.

Discussion. The practical implications of the data presented in this paper will first be considered. Study of the measles virus during the last 3 years has been somewhat hampered by lack of large quantities of susceptible normal human cells free of contaminating agents. The finding reported here that the virus can be adapted to human amnion cells in which it induces characteristic cytopathic changes repairs this deficiency.

The results showing that at least one strain of virus can be propagated serially in the developing chick embryo may serve to remove any remaining doubts of the susceptibility of this host to the agent of measles. This demonstration is of importance, we believe, because of the many advantages the living chick

embryo or cells derived from it‡ would seem to offer in comparison with other media as a source of virus for the preparation of vaccines.

In considering the possibility of vaccination against measles, we may also emphasize the fact that the presence and amount of virus in chick embryonic materials can now be reliably and conveniently determined by means of tissue culture technics. Accordingly, attempts to immunize man against measles with egg-adapted virus can again be undertaken under more precise conditions of control than were available to workers in the past who have been concerned with this problem.

Several phenomena of more general biologic interest are presented by these observations. For example, recognition of a second and distinct cytopathogenic effect of the virus on human amnion cells has led us to inquire concerning the factors responsible for its appearance. Experiments are in progress to determine whether capacity to induce the spindle cell transformation is an original characteristic of the virus as it occurs in nature, or whether it emerged, possibly as a mutation, during the continued passage of the agent under the artificial conditions of the tissue cultures. Reissig, Black and Melnick(14) have recently shown with the Edmonston strain in cultures of human carcinoma cells that the cytopathic changes vary from the syncytial to the spindle form depending upon concentration of glutamine in the medium. At present, we believe that the phenomenon we have observed does not depend on this mechanism, since composition of the medium has remained constant, while the cytopathogenicity of the virus has varied.

The spindle cell transformation, as contrasted with the formation of syncytia, is also noteworthy, since it shows that wide departures from the "normal" or expected effect of a virus may occur within the same cell system. Indeed, in the case of measles virus these expressions of cytopathogenicity are so different that for a time we considered the possibility that the spindle cell change might have been induced by an unrelated agent introduced as a contaminant.

The factors which condition multiplication of the virus in the chick embryo have not yet been clearly defined. In the successful experiments, in contrast with those that failed, a longer period of incubation was allowed and another route of inoculation was employed. Furthermore, the virus employed as original inoculum had been subjected to many more passages in tissue cultures—an experience that may have increased its capacity to multiply in the chick embryo. Experiments have been undertaken to determine whether proliferation of the virus is dependent on any of these modifications.

Summary. 1. A strain of measles virus originally isolated in cultures of human renal cells has been propagated throughout 28 serial passages in cultures of human amnion cells. In the latter system it induces 2 types of cytopathic change: (1) formation of "syncytia" or "multinuclear giant cells" in which intranuclear and intracytoplasmic inclusions are prominent features; (2) only recently recognized, the assumption by individual epithelial cells of a characteristic fusiform or stellate configuration. In certain of these affected cells eosinophilic intranuclear inclusions are present that resemble those found in the nuclei of the syncytia. Eventually both types of change terminate in cellular necrosis and disintegration. In contrast to the dual response of amnion cells only the formation of syncytia has been observed in cultures of renal cells infected with the virus. 2. The Edmonston strain of measles virus from the 28th passage in human amnion cells was inoculated into chick embryos. In this host it has been maintained throughout 12 successive passages. Multiplication of the agent was demonstrated by addition of chick embryonic materials to cultures of human amnion cells. This procedure was necessary since no definite indication of viral activity has as yet been distinguished within the egg. 3. The virus present in the 9th chick embryo passage was identified as the measles agent in complement fixation

‡ Recently multiplication of the egg-adapted virus has been demonstrated throughout 6 serial passages in cultures of chick embryo cells. Details of these experiments will be published later.

and virus-neutralization tests with acute and convalescent phase measles sera.

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Decreased Oxygen Need as a Factor in Anemia of Hypophysectomized Animals.* (23141)

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Hypophysectomy induces an anemia in all animals studied, including the human. Various therapies have been shown to prevent this anemia in the rat, success being obtained with thyroxin and androgen(1), thyroxin, androgen, and a high protein diet(1,2), thyroxin and cortisone(3,4), adrenocorticotropic hormone(5), "erythropoietic hormone"(6,7), and cobalt(8,9). In spite of these results, the reason hypophysectomy induces an anemia is still under discussion. Some have claimed that loss of a pituitary erythropoietic factor explains it(6,7), while others have claimed that hypothyroidism and hypoadrenocorticalism, which inevitably follow hypophysectomy, are playing a role(10,11,3,4), although the pituitary seems to be involved in some manner which is in addition to its role in regulating thyroid and adrenal glands(11). Recent work whereby a combined therapy of growth hormone, thyroxin and cortisone returned all aspects of post-hypophysectomy anemia to near normal values‡ has indicated that growth hormone may be involved as well as thyroid and adrenal hormones.

These findings, however, do not explain the underlying mechanism of post-hypophysectomy anemia. The fact that oxygen consumption is decreased after hypophysectomy (12,13,14) and that anoxic anoxia is still considered to be the fundamental stimulant for erythropoiesis induced us to attempt to determine whether this anemia was correlated in any way with a decreased need for oxygen in hypophysectomized rats.

Methods. Unless otherwise indicated, adult female rats of the Wistar strain were used. All rats were fed Purina Chow *ad libitum* supplemented once a week with lettuce. Completeness of hypophysectomy was checked by observing the sella turcica with a binocular dissecting microscope at autopsy. Erythrocyte counts were done in duplicate using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin deter-

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‡ Unpublished data.

TABLE I. Blood Picture and Oxygen Consumption in Hypophysectomized Adult Female Rats before and after a Blood Transfusion. Average amount of blood transfused was 4.5 cc.

	Normal controls	Hypophysectomized rats — Before transfusion		After transfusion	
	13 rats	7 rats	% of normal	7 rats	% of normal
Erythrocyte count in millions/mm³	8.21 ± .16	6.99 ± .17	86	9.39 ± .36	114
Hematocrit, %	46.3 ± .75	36.3 ±1.10	79	48.1 ±1.60	103
Hemoglobin, g/100 cc	14.9 ± .23	13.2 ± .28	89	16.3 ± .46	109
Oxygen consumption, l/m² body surface/hr	7.79 ± .22	5.26 ± .17	68	4.71 ± .24	61

± = Stand. error.

minations were made on a Klett-Summerson photoelectric colorimeter, and hematocrit readings were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 rpm. Oxygen consumption was measured as follows: rats were placed in the lower chamber of a 200 mm Frühling and Schultz desiccator; the upper chamber contained wire baskets of Ascarite and Dehydrite for absorption of Co_2 and water respectively. This desiccator was connected with a 500 ml linear or volume recording spirometer which, in turn, was attached to the oxygen supply. Oxygen was placed in the spirometer; the amount used by the rat was recorded directly on a kymograph placed beside the spirometer. Rats were allowed a 30 minute stabilizing period before measurements were started, the oxygen consumed being recorded for 30 minutes with the animal at rest; the figure obtained was then corrected to standard temperature and pressure, and the final result expressed as liters consumed/m^2 of body surface/hour according to the following formula (15): $\text{l/sq. meter/hr} = \frac{v}{t} \times \frac{P}{760} \times \frac{273}{T} \times \frac{10{,}000}{(wt)^{2/3} \times 10} \times \frac{1}{1000}$, where $\frac{v}{t}$ = ml of oxygen used over time in hours, P = barometric pressure, T = temperature in the desiccator, $\frac{10{,}000}{(wt)^{2/3} \times 10}$ a means of converting weight to surface area, and $\frac{1}{1000}$ simply a means of converting to liters. The temperature inside the desiccator showed very little change. As fasting hypophysectomized animals for 12 hours occasionally induced a hemoconcentration, fasting had to be avoided. These results are, therefore, subject to the error that the process of digestion might induce.

Procedure and results. The experiment was designed (1) to establish that hypophysectomy actually induces a decrease in oxygen consumption, (2) to determine whether such a decrease, if confirmed, was the result of the anemic condition of the animals, and if not, (3) to attempt to elevate oxygen consumption to see what effects it would have on the blood picture. To confirm that hypophysectomized animals consume less oxygen than normal animals, adult female rats were hypophysectomized and left unmolested for 60 days. At this time the rats were anesthetized with ether and blood removed from the tail for analysis; this was followed by an oxygen consumption determination.

The results are shown in Table I. Hypophysectomy induced an anemia exhibiting a 14% decrease in erythrocyte count, 21% decrease in hematocrit, and an 11% decrease in hemoglobin; the oxygen consumption was 5.26 l/hour in contrast to the value for normal rats of 7.79 l, a 32% decrease.

To determine whether this decrease in oxygen consumption was due to the anemia, these same hypophysectomized rats were given a transfusion of an average of 4.5 cc of whole blood via tail vein. This was followed by an oxygen consumption determination and blood study. Table I shows that the erythrocyte count, hematocrit reading and hemoglobin values were returned to normal by the transfusion; in spite of this, the oxygen consumption remained at a level which was only 61% of normal. These results showed that the

TABLE II. Effects of Various Treatments on Blood Picture and Oxygen Consumption of Adult Female Rats.

Treatment	# rats	Erythrocyte count, millions/mm³	% of normal	Hematocrit, %	% of normal	Hemoglobin, g/100 cc	% of normal	Oxygen consumption, l/m²/hr	% of normal
Normal controls—no treatment	52	7.97 ± .13	100	43.8 ± .50	100	15.0 ± .31	100	8.74 ± .26	100
Thyroidectomy + adrenalectomy 60 days after surgery	6	6.56 ± .21	82	33.2 ±1.24	76	11.3 ± .21	75	5.28 ± .44	60
Hyp. for 50 days followed by .005 mg thyroxin + .6 mg cortisone for 60 days	8	8.44 ± .14	106	46.2 ± .74	105	15.5 ± .25	103	8.44 ± .72	97
*Hyp. for 75 days followed by growth hormone for 50 days	15	6.67 ± .12	84	37.1 ± .5	85	12.4 ± .2	83	6.43 ± .20	74

* Sprague-Dawley rats. Growth hormone (Armour's lyophilized lot #R377237) given in gradually increasing doses starting with 0.2 mg and ending with 0.8 mg.

± = Stand. error.

hypophysectomized rat does not consume less oxygen because of the anemia for it continued to utilize a decreased amount of oxygen when the erythrocyte picture was normal.

Extensive work was done using dinitrophenol to elevate oxygen consumption without affecting general metabolism; it was hoped that this would furnish direct evidence that oxygen need on the part of the hypophysectomized animal was regulating the rate of erythropoiesis. This material proved to be very toxic to hypophysectomized rats when injected chronically; our experiments with this substance were failures in that small doses were ineffective and large doses lethal.

Since the idea of a possible correlation between post-hypophysectomy anemia and oxygen need has been in mind for some time, oxygen consumptions were determined in several recent experiments. These results are shown in Table II. The dual surgery of thyroidectomy and adrenalectomy induced an anemia which was similar to that found after hypophysectomy, and the oxygen consumption was reduced to 60% of normal. Table II also shows that a treatment such as daily injections of 0.005 mg of thyroxin and 0.6 mg of cortisone for 60 days will eliminate post-hypophysectomy anemia; at the same time oxygen consumption was also returned to normal levels. In addition, daily injections of gradually increasing doses of growth hormone (0.2 mg to 0.8 mg) over a 50-day period had no effect on the peripheral blood picture and no effect on oxygen consumption.

Discussion. The hypophysectomized animal becomes anemic and this anemia is accompanied by a decrease in oxygen consumption. Is this decrease in oxygen consumption a result of the anemia or is the anemia simply a reflection of the decreased need for oxygen by the hypophysectomized rat which, in turn, is reflected in a decreased erythropoiesis? The fact that the hypophysectomized rat does not utilize a normal amount of oxygen when its blood picture is restored to normal supports the theory that the hypophysectomized animal is anemic simply because fewer erythrocytes provide an adequate oxygen and Co_2 transport. In addition, procedures such as combined thyroidectomy and adrenalectomy

induce a concurrent decrease in oxygen consumption and erythrocyte count; therapy which had no effect on the peripheral blood picture, such as growth hormone, had no effect on oxygen consumption and a combined thyroxin-cortisone therapy, which eliminated the anemia, elevated oxygen consumption to normal levels. The above evidence is admittedly indirect in character; direct evidence, which might be obtained by elevating oxygen consumption (without increasing general metabolism) and concurrently stimulating erythropoiesis, is difficult to obtain in hypophysectomized animals.

Other features of post-hypophysectomy anemia are in agreement with this theory. Erythropoiesis does occur in hypophysectomized animals, as can be shown in several ways. (1) The erythrocyte count, hematocrit, and hemoglobin do not show a continued decrease after hypophysectomy; 30 to 40 days after surgery the blood picture plateaus, remaining approximately at this level for as long as one year after hypophysectomy. (2) Although Meyer, *et al.*(16) could not stimulate erythropoiesis in bone marrow of hypophysectomized rats by decreasing oxygen tension, Feigin and Gordon(17) were able to do so by decreasing oxygen tension to a greater degree. (3) Querido and Overbeek (18), Finkelstein, *et al.*(19), and Silbergleit (20) have all shown that the hypophysectomized rat can respond to bleeding by an increased rate of erythrocyte formation and subsequent increase in reticulocytes, the counts returning to a level typical of unbled hypophysectomized animals. (4) Baker, *et al.*(21) have reported that hypophysectomized rats treated with total x-radiation became more anemic than untreated hypophysectomized rats, but soon recovered to such a degree that the blood picture was typical of the untreated hypophysectomized animal. All of these experiments indicate that erythropoiesis does occur in the marrow of the hypophysectomized rat; in addition, several of these studies indicate that the bone marrow in these rats will respond to alterations in oxygen supply and demand if the stimulus is severe enough.

This theory that a decreased oxygen need reduces rate of erythropoiesis in the hypophysectomized rat has been used by Fried, *et al.* (22) to explain erythropoietin production. The amount of plasma factor may very well be the intermediary between oxygen need on the part of the tissues and the bone marrow response.

The present experiment does not prove that decreased oxygen need is the sole cause of the anemia which follows removal of the hypophysis but the evidence is plentiful and cannot be ignored. There is no doubt but that erythropoiesis can occur in the absence of the pituitary gland. If this theory is correct, the endocrine glands do not influence erythropoiesis directly, as is suggested by sponsors of the erythropoietic hormone, but merely influence the rate of red cell production by their control over general metabolism and the concomitant changes in oxygen need by the tissues.

Summary. Adult female rats were hypophysectomized and studied 60 days later; they exhibited the usual post-hypophysectomy anemia. The theory that the anemia in the hypophysectomized animal is simply a reflection of a decreased need for oxygen is supported by the finding that oxygen consumption was decreased in these animals and that it remained at a low level even when the peripheral blood picture was restored to normal via transfusion of whole blood. The theory is further supported by the finding that procedures which altered the erythrocyte level caused a similar change in oxygen consumption, and by much evidence indicating that the bone marrow can function normally in the absence of the pituitary gland.

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Estimation of Amount of Oxytocin Released as Result of Nursing Stimuli in Lactating Rat.* (23142)

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There seems to be little doubt that the posterior lobe of the pituitary gland plays an important role in milk let-down(1). It is also generally accepted(2-4) that oxytocin from this gland is responsible for contraction of myoepithelial elements surrounding alveoli and small ducts of the mammary gland thus making available milk for withdrawal. Amount of oxytocin required by various species for milk let-down has been investigated to some extent for the rabbit(5-8), sheep(9), goat(9,10), sow(11) and human(12). However, the amount required by the rat has not been adequately determined. Folley(10), reporting upon unpublished work by Cowie, found that young of anaesthetized lactating rats were able to obtain milk, as determined by test weighing, after a single i.p. injection of .025 I.U. oxytocin into the mother. Grosvenor and Turner(13) found that .2 I.U./kg subc. restored milk let-down in lactating rats in which normal let-down had been inhibited by ergotamine treatment.

In the present study the amount of oxytocin required for normal milk let-down in lactating rats has been determined by injecting various levels of oxytocin i.v. into anaesthetized mothers and comparing weight of milk obtained by the young with normal values previously obtained(14).

Materials and methods. Forty-one lactating rats, each with its first litter and weighing 190-320 g, were housed in individual cages and fed Purina Lab Chow and water *ad libitum.* Shortly after birth each litter was reduced to 6 young and when 14 days old was isolated from the mother for 10 hours. Each mother was weighed and injected with Nembutal (3 mg/100 g) i.p. 10-20 minutes prior to end of isolation period. When completely anaesthetized, she was laid on her side and her litter replaced. Twenty-seven animals received oxytocin‡ in dose of .02, .05 or .1 USP/kg injected i.v., in a volume not exceeding .06 ml, into the superior epigastric vein a few minutes after the young were replaced and while they were actively sucking.

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† Postdoctoral Fellow of N.I.H.

‡ We are grateful to Dr. I. Bunding of Armour Laboratories for the supply of oxytocin.

TABLE I. Milk Removal by Litters of Anaesthetized Nursing Rats Injected with Oxytocin on 14th Day Postpartum.

Treatment	No. of rats	Avg wt of litters (g)	Avg wt of milk (g)	Avg % Wt of milk / Wt of litter
Control*	40	144.0	5.5	3.8 ± .19
Nembutal	14	164.5	.0	.0
Nembutal + .02 USP/kg oxytocin	11	159.0	4.7	3.0 ± .82
Nembutal + .05 USP/kg oxytocin	11	164.2	6.3	3.8 ± .20
Nembutal + .1 USP/kg oxytocin	5	168.8	9.8	5.9 ± .45

* Data from Grosvenor and Turner(14).

Eight similarly received .06 ml distilled water; the remaining were uninjected. After each litter had been nursed for 30 minutes they were removed, weighed, killed by decapitation and stomach contents removed and weighed.

Results. Nembutal anaesthesia blocked the let-down reflex in lactating rats and prevented removal of milk by young during 30 minutes nursing (Table I). Oxytocin injected i.v. a few minutes after nursing had commenced, resulted in milk let-down after a latent period of 5-10 seconds and seemed to persist 4-5 minutes. These times were obtained by observing the young; when milk let-down had started the young immediately began sucking actively. After milk flow had apparently ceased they became quiet and sucked only intermittently during the remainder of the experiment. Amount of milk obtained expressed as percent litter body weight secured during 30 minutes by young of anaesthetized lactating rats injected with oxytocin is shown in Table I. Values obtained with doses of oxytocin of .02, .05 and .1 USP/kg were 3.0 ± .82, 3.8 ± .20 and 5.9 ± .45, respectively. Control injections of distilled water were without effect.

Discussion. Our previous results(14) obtained with 40 litters, each with 6 young, on 14th day postpartum, indicated that weight of milk in stomachs of young expressed as percent litter body weight obtained after 30 minutes nursing following 10 hours isolation gave a mean of 3.8 ± .19. Results of the present study obtained under the same standard conditions indicated .05 USP/kg oxytocin was necessary to evoke let-down of same mean quantity of milk whereas .02 USP/kg was insufficient. Since in the previous study (14) 12 of 40 normal rats showed milk let-down permitting removal of 2.0-3.0%, 15 rats of 3.7% and 12 others a removal of 4.6-5.6%, it is suggested, on the basis of present results, the amount of oxytocin released by those animals was approximately .02, .05 and .1 USP/kg. It is concluded that normal 14 day postpartum lactating rats vary in amount of release of oxytocin following nursing stimuli from less than .02 to .1 USP/kg. It is of interest to note injection of .1 USP/kg oxytocin elicited a much greater withdrawal of milk which would seem to indicate more milk is present in mammary glands of lactating rats than a litter of 6 normally obtains during 30 minutes nursing, although previous observations(14) indicated young were satiated by this time.

That complete anaesthesia blocks milk let-down reflex and that posterior pituitary principles are able to cause discharge of milk in the anaesthetized animal has been determined for species other than the rat(6-8,10,15). These observations have been extended in the present work to include the rat. Extensive experiments on the lactating sow have led Whittlestone(11) to estimate 0.5-1.0 I.U. oxytocin released normally at a nursing. Cross(8) found .05 I.U. similarly needed for the lactating rabbit. Results of this study indicate about .014 USP oxytocin is released per lactating rat in response to nursing stimuli which, on a body weight basis, is approximately 3 and 10 times the amount released by the rabbit and sow, respectively.

Summary. We estimated amount of oxytocin required for normal milk let-down in lactating rats. This was determined by in-

jecting various doses of oxytocin i.v. into anaesthetized mothers while young were actively sucking and comparing weight of milk obtained by young expressed as percent litter body weight with normal values. Nembutal (3 mg/100 g) blocked milk let-down reflex and prevented removal of milk by the young. .05 USP/kg oxytocin restored normal milk withdrawal; .02 USP/kg did not. Much greater yields were obtained with .1 USP/kg. Milk flow commenced with all doses after latent period of 5-10 seconds and lasted 4-5 minutes. It is concluded that normal 14 day postpartum lactating rats vary in amount of release of oxytocin following nursing stimuli from less than .02 to .1 USP/kg.

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Antigenicity of Egg White Proteins in the White Mouse.* (23143)

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It has been well demonstrated by a variety of technics(1-3) that diverse antibodies other than anti-ovalbumin are present in "specific" antisera directed against crystalline ovalbumin. Indeed, Vaughan and Kabat(4) have demonstrated that, in addition to anti-conalbumin, anti-ovomucoid and anti-lysozyme, antibodies exist in such sera as a result of the antigenic stimulation of hitherto unidentified egg white impurities in ovalbumin. In attempting to relate serum antibody titers to fatal anaphylactic shock in the ovalbumin-sensitized mouse the question of antibody identity arose. Having previously noted(5) that all proteins do not manifest the same ability to sensitize the white mouse to the same degree, the sensitizing capacities and cross-reactivities of the available protein contaminants of crystalline ovalbumin were characterized by the pertussis-technic(6).

Materials and methods. Thrice recrystallized egg albumin (Ea) was prepared according to the procedure of Kekwick and Cannan (7) as described by Kabat and Meyer(8). Conalbumin (Ca), as the crystalline iron complex, and ovomucoid (Ovo) were prepared(9) and generously supplied by Dr. Robert C. Warner of New York University. Crystalline lysozyme (Lyso) was purchased from Nutritional Biochemicals Corp. Female white mice of the Swiss-Webster strain weighing about 20 g were obtained from Tumblebrook Farms. Groups of animals were sensitized by a single intraperitoneal injection of a given amount of the egg white protein contained in 0.5 ml of a suspension of 2 x 10^{10}

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TABLE I. Challenging Antigen Administered Intravenously 10 Days after Intraperitoneal Sensitization.

Challenging Antigen	mg	Sensitizing antigen: Ea 1 mg	Ea 5 mg	*H. pertussis*-Ea 1 mg	*H. pertussis*-Ea 5 mg	Ovo .5 mg	Ovo 1 mg	*H. pertussis*-Ovo .5 mg	*H. pertussis*-Ovo 1 mg	Ca, .5 mg	*H. pertussis*-Ca, .5 mg	Lyso, 1 mg	*H. pertussis*-Lyso, 1 mg
Ea	1	1/ 6*		17/21						0/10	3/15		
"	5						0/7		1/10			1/13	5/14
Ovo	.1	0/21		0/19		0/14	0/9	0/10	0/13				
"	.3			0/ 4			0/8	0/ 4	0/12			0/13	0/14
Ca	.1	0/14	0/10	0/13	6/12		0/7		0/10	0/10	11/11	0/13	1/14
Lyso	.1											0/ 1	0/ 4
"	1		0/18		0/18				0/18		0/17	0/15	14/16

* Values expressed as No. of animals dead of anaphylactic shock over No. of animals challenged.

Hemophilus pertussis phase I organisms. Control groups received the same quantity of antigen in saline solution without the bacteria. Ten days after this sensitizing dose the mice of each group were challenged by the intravenous injection of either the homologous antigen or one of the heterologous egg white proteins.

Results. The data in Table I are expressed in terms of ratio of number of animals dead of anaphylactic shock to number of animals challenged. No attempt was made to record severity of the anaphylactic reaction but for this "all-or-none" effect.

It is seen that Ovo is not capable of sensitizing the white mouse by this technic in contradistinction to both Ca and Lyso. It is also to be noted that whereas Ca produces anaphylactic death in the mouse sensitized to Ea, Lyso does not in the amounts used for challenge. On the other hand, Ea elicits shock in mice sensitized to Lyso and Ca. How much of this interrelationship depends on the integral amounts of Ca and Lyso present in Ea is not known for no attempt was made at quantitation by varying the sensitizing and challenging dose. It seems reasonable to assume from these experiments that (1) either there is more Ca than there is Lyso as an impurity in Ea, or (2) Ca is a far stronger antigen than Lyso. At any rate, from the relative amounts of antigen used in the challenge it would seem to indicate that the amount of Ca and Lyso producing shock need be far greater than that present in the Ea used.

These data offer an *in vivo* conformation of the results of Vaughan and Kabat(10) wherein relatively greater amounts of anti-Ca was found in anti-Ea as compared to antibodies to the other trace impurities. It was also indicated(10) that antibodies to impurities were demonstrable even though minimal quantities of antigen were used for immunization. In the present study, anti-Ca was demonstrated in those mice sensitized with 5 mg Ea as a single dose and not in those receiving 1 mg Ea.

The enhancing effect of pertussis is well demonstrated, be it by adjuvant action or by some other mechanism. Antibodies to trace impurities have been demonstrated where very large quantities of purified antigen have been used for immunization(1), Freund adjuvant has been utilized(2) or alum-precipitated antigen has been given(10) to mention several technics. The mechanism of pertussis action has not yet been well categorized.

Summary. Conalbumin and lysozyme are effective antigens in producing sensitivity in pertussis-treated white mice. Conalbumin cross-reacts with mice sensitized to ovalbumin, lysozyme does not. Ovalbumin shocks mice sensitized to both conalbumin and lysozyme, however. Ovomucoid is not antigenic.

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Anticancer Activity of Purine Antagonists and Their Ribosides. I. Comparative Studies with 6-Mercaptopurine and 6-Mercaptopurine Riboside.* (23144)

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Synthesis of 6-mercapto-9-β-D-ribofuranosylpurine (6-MP-riboside) has been achieved in 70-80% yields by reaction of 6-chloro-9-β-D-ribofuranosylpurine with hydrogen sulfide in methanolic sodium methoxide(1). This "fraudulent" riboside was considered worthy of synthesis and trial against experimental neoplasms in view of the well-established antibacterial(2) and antileukemic(3) activity of 6-mercaptopurine (6-MP). The possibility exists that 6-MP may be converted to 6-MP-riboside or ribotide before it becomes an active antimetabolite, and it has been noted that certain 6-MP-resistant bacteria lack the ability to convert hypoxanthine to purine ribotides. To obtain preliminary information on the potential usefulness of 6-MP-riboside, experiments have been carried out to test: (a) Comparative activity of 6-MP and 6-MP-riboside as inhibitors of growth of Adenocarcinoma 755, an experimental neoplasm of unusual sensitivity to known purine antagonists (4). (b) Sensitivity of *Streptococcus faecalis* (ATCC No. 8043, SF/O) and 6-MP-resistant lines of this bacterium to inhibition by 6-MP-riboside. (c) Effects of 6-MP-riboside on growth of a 6-MP-resistant line of Adenocarcinoma 755.

The results obtained in such studies are reported herein.

Inhibition of Adenocarcinoma 755. Highly inbred C57 black mice obtained from Jackson Memorial Laboratories were implanted with Adenocarcinoma 755 by the usual trocar method, randomized, and broken into groups of 10 mice each. The comparative inhibitory effects of 6-MP and 6-MP-riboside on growth of this tumor were then determined. Both compounds were administered intraperitoneally; therapy was initiated 24 hours after tumor implantation and continued once a day (Schedule B) or 3 times daily (Schedule C) for 11 or 12 days. All tumors were excised and weighed individually on the 12th and 14th day. The results of these inhibition assays are summarized in Table I.

Effects of 6-Mercaptopurine and 6-Mercaptopurine Riboside on Growth of Streptococcus faecalis. To learn something of the cross-resistance in biologic systems between 6-MP and its riboside, experiments were carried out in which *S. faecalis* (SF/O) and two 6-MP-resistant lines of *S. faecalis* (SF/MP(5) and SF/MPcc†) were compared as to their susceptibility to growth inhibition by 6-MP and its riboside. A modification of the medium of Flynn, *et al.*(6) was used in these experi-

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† This resistant line was isolated by the same procedure as was SF/MP(5).

TABLE I. Comparative Effects of 6-Mercaptopurine and 6-Mercaptopurine Riboside on Growth of Adenocarcinoma 755.

Exp. No.	Treatment	Dosage mg/kg/day	Dosage mM/kg/day	Avg animal wt change (g)	Mortality (out of 10)	Avg tumor wt, % of control	Dosage schedule
1	6-MP	30	.20	+1	2	2	B
		15	.10	+1	1	3	B
		7.5	.05	+2	1	3	B
	6-MP-riboside	70	.25	+1	0	2	B
		35	.13	+1	0	3	B
		17	.07	+1	0	2	B
2	6-MP	15	.10	−2	4	0	C
		7.5	.05	−1	2	0	C
		3.8	.025	−1	0	0	C
		1.9	.012	0	1	15	C
		.9	.006	+1	0	79	C
		.45	.003	0	0	88	C
		.10	.0006	+1	0	96	C
	6-MP-riboside	17	.07	−3	3	0	C
		8.5	.035	−2	1	0	C
		4.3	.018	-2	1	3	C
		2.1	.009	0	2	47	C
		1.1	.005	+1	1	84	C
		.25	.001	0	0	84	C

Note: Therapy was initiated 24 hr following tumor implantation and continued once daily for 11 days (B schedule) or 3 times daily for 12 days (C schedule). Tumors were weighed on 12th day (B schedule) or 14th day (C schedule).

ments. Adenine, guanine, xanthine, and uracil were omitted from the medium. This purine-pyrimidine-free basal medium was supplemented with pteroylglutamic acid, 3 mγ/ml, and sodium acetate, 100 γ/ml. To avoid exposure of 6-MP or 6-MP-riboside to heat, all solutions and medium were filtered through ultrafine sintered glass filters and added aseptically to sterile tubes. The inoculum for each tube consisted of 0.1 ml of a twice washed culture which contained approximately 1.5 x 10^6 cells. Growth was measured as optical density at 660 millimicrons in a Coleman Spectrophotometer after 22 hours of incubation at 35°C. The results of this study are summarized in Table II.

Effects of 6-MP-Riboside on a 6-MP-Resistant Line of Adenocarcinoma 755. Although microbiologic studies showed that 2 different 6-MP-resistant lines of *S. faecalis* were solidly cross-resistant to 6-MP-riboside, it was

TABLE II. Effects of 6-Mercaptopurine and 6-Mercaptopurine Riboside on Growth of *S. faecalis*.

Inhibitor (γ/ml)	Optical density at 22 hr: 6-MP SF/O	6-MP SF/MP	6-MP SF/MPcc	6-MP-riboside SF/O	6-MP-riboside SF/MP	6-MP-riboside SF/MPcc
500	0	0	0	0	0	.07
150	.10	.47	.23	.04	.53	.29
50	.25	.52	.30	.05	.48	.38
15	.25	.42	.32	0	.48	.30
5	.18	.38	.30	0	.47	.27
1.5	.19	.38	.30	0	.48	.24
.5	.08	.38	.23	0	.48	.25
.15	0	.39	.29	0	.47	.27
.05	0	.38	.29	0	.48	.28
.015	.21	.39	.30	.23	.51	.32
.005	.34	.40	.30	.33	.49	.32
.0015	.34	.40	.29	.35	.49	.32
.0005	.36	.42	.30	.35	.50	.33
0	.34	.49	.36	.36	.47	.31

TABLE III. Effects of 6-Mercaptopurine Riboside on Growth of a 6-MP-Resistant Line of Adenocarcinoma 755 (755/MP).

Exp. No.	Tumor	Treatment	Dosage (mg/kg/day)	Avg animal wt change (g)	Mortality (out of 10)	Avg tumor wt, % of untreated controls
1	755	6-MP	20	− .7	1	1
	755/MP		20	+ .1	0	59
	755		10	− .5	0	5
	755/MP		10	+2.1	0	88
	755		5	+ .1	1	13
	755/MP		5	+3.0	0	96
	755	6-MP-riboside	34	−1.0	0	3
	755/MP		34	+2.5	0	100
	755		17	−1.9	1	1
	755/MP		17	+2.6	0	100
2	755	6-MP	20	− .9	1	.6
	755/MP		20	−3.2	5	34
	755		10	+ .7	0	2
	755/MP		10	− .1	1	46
	755	6-MP-riboside	34	+ .9	0	1
	755/MP		34	−2.4	2	26
	755		17	+ .4	1	2
	755/MP		17	− .5	0	48

Note: Therapy initiated 24 hr after tumor implantation and continued daily for 11 days. Tumors weighed on 12th day.

considered worthwhile to assay this riboside against a 6-MP-resistant neoplasm.

Recently we have selected a 6-MP-resistant line of Adenocarcinoma 755 (755/MP) by serial passage of this tumor in C57 mice treated with 6-MP at levels of 20-40 mg/kg 3 times weekly. After 8 such passages this line of 755 was assayed for response to 6-MP and shown to be significantly resistant.

In additional experiments, the cross-resistance of 755/MP to 6-MP-riboside was studied, Table III.

Discussion. The data presented in Tables I and III clearly indicate that ribosidation of 6-mercaptopurine does not negate the inhibitory activity of this antagonist against Adenocarcinoma 755. Sufficient quantities of 6-mercaptopurine riboside have not yet been available to establish conclusively the relative chemotherapeutic index of this compound for comparison with unsubstituted 6-mercaptopurine; however, the data presented in Table I suggest that the 2 compounds are of the same order of activity. In Experiment No. 2, Table I, the lowest level of 6-MP showing significant activity was 0.012 mM/kg/day (approximately 2 mg/kg/day); 6-MP-riboside significantly inhibited growth of Adenocarcinoma 755 at a level of 0.018 mM/kg/day. These preliminary screening results would not suggest any practical advantage of 6-MP-riboside over 6-MP.

6-Mercaptopurine riboside was synthesized with the hope that it might be inhibitory toward 6-MP-resistant cells. That this hope was not realized may be seen in Table II which shows that 2 different 6-MP-resistant lines of *S. faecalis* are cross-resistant to the riboside and Table III which shows that a 6-MP-resistant line of Adenocarcinoma 755 is cross-resistant to 6-MP-riboside.

The effects of 6-MP and 6-MP-riboside on growth of SF/O show some interesting differences. On a weight basis the 2 compounds have comparable activity with 50% inhibition at 0.03 γ/ml. As concentration of 6-MP was increased to 50 γ/ml growth slightly less than the control occurred, finally with higher concentrations inhibition of growth was again observed. No such growth response was observed with 6-MP-riboside.

It is possible that 6-MP contains a very small amount of hypoxanthine and as the appropriate ratio of purine and antagonist ap-

pears in the medium, growth occurs or growth inhibition is observed. However, chromatographic examination of the 6-MP indicated less than 1% contamination with hypoxanthine. The trough of the response curve disappears after 48 hours incubation, at which time growth in concentrations up to 150 γ/ml is comparable to the control. Inhibition of the riboside is also overcome within a 120-hour incubation period. An alternative explanation for the 6-MP results is that 6-MP over a narrow concentration range serves as a purine source (after conversion to hypoxanthine) and thus stimulates growth and overcomes to a degree unchanged antagonist at certain concentrations. Inhibition by low levels in the early phase of growth may be due in some way to interference with the *de novo* formation of coenzymes or precursors necessary for biosynthesis of nucleic acids.

The present results require considerable extension prior to any final conclusion regarding the possible value of 6-mercaptopurine riboside as a chemotherapeutic agent; however, to date no evidence has been obtained which would suggest any advantage over the much more easily prepared 6-mercaptopurine.

Observation of cross-resistance of 6-mercaptopurine-resistant bacterial and neoplastic cells to 6-MP-riboside deletes one theoretical explanation of the mechanism of 6-MP-resistance—that is, that such resistant cells have lost the ability to ribosidate 6-MP.

Summary. (1) 6-Mercaptopurine riboside has been assayed for inhibitory activity against Adenocarcinoma 755 in mice and has been found to be of approximately the same activity as 6-mercaptopurine. (2) Lines of *S. faecalis* resistant to 6-mercaptopurine have been found to be cross-resistant to 6-mercaptopurine riboside. (3) A line of Adenocarcinoma 755 selected for resistance to 6-mercaptopurine showed cross-resistance to 6-mercaptopurine riboside.

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Influence of Thiourea on Growth of Cells of Midbrain in Frogs.* (23145)

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Recent studies demonstrate that thyroid hormone affects the differentiation of particular cells of the amphibian central nervous system. Following treatment with thyroxine, certain cells of the medulla oblongata grow (1,2). Cells of the lateral motor column increase their nuclear size in response to both direct and indirect thyroxine stimulation(3). Several independent lines of investigation have shown that the level of differentiation attained by cells of the mesencephalic nucleus of the trigeminal nerve in tadpoles of *Rana pipiens* is *directly* dependent upon concentration of thyroid hormone available to those cells(4). From these studies it is inferred that blockage of normal metamorphosis by treatment of tadpoles with thiourea might result in termination of growth of the cells of the mesencephalic V nucleus, and perhaps even in their involution. Moreover, treatment of the metamorphosed frog with

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thiourea might reveal the extent to which these specialized midbrain cells are under thyroid hormone control in the postmetamorphic period. The present study reports the testing of these inferences.

Material and methods. All animals were reared from eggs laid in the laboratory (*Xenopus laevis*) or in ponds near Iowa City (*Rana pipiens*). Stock animals were kept in groups in aquaria, while experimental and control tadpoles were kept in individual bowls in 125 ml of water. Metamorphosed Xenopus were kept individually in covered dishes with 300-500 ml of water. Thiourea was added to the water of the bowls to attain concentrations of either 0.033%(5) or 0.05-0.06% for Rana, and 0.25-0.30% for Xenopus larvae and adults. In order to permit the initial spurt of rapid mesencephalic V nucleus cell growth, which occurs late in the larval period(6), treatment was deferred until the tadpoles were within 5-10 days of the time of expected emergence of the forelimbs(7,8). Control animals were selected to be about the same age, stage and size as the experimental animals at completion of the thiourea treatment. On the average the control animals were very slightly shorter than the matching thiourea treated partners. This discrepancy could bias the results only in the sense of decreasing the anticipated differences in cell size, since there is a positive correlation between body size and mesencephalic V nucleus cell size(6). Fixation of the animals, preparation of the slides, and selection and measurement of at least 75 cells on one side of each animal were carried out as before(4,6). The significance of differences between treated and control animals was determined by the method of paired comparisons (related measures).

Results. Rana. Treatment of 25 tadpoles with 0.033% thiourea failed to inhibit metamorphosis. These animals were discarded. A similar group immersed in 0.05-0.06% thiourea yielded 4 animals in which metamorphosis was completely blocked after 8 or more days, and 4 others in which metamorphosis was significantly slowed. In the 4 animals showing metamorphic stasis, and in 2 of the others, the sizes of cells and nuclei of the mesencephalic V nucleus were smaller than in control tadpoles (Table I).

TABLE I. Thiourea Treatment Time of *Rana pipiens*, and Stage at Fixation, with Average Sizes of Cells and Nuclei, in μ^2, Compared to Those of Non-Treated Animals.

Days in thiourea	Fixation stage (7)	Experimental		Control	
		Cell*	Nucleus*	Cell*	Nucleus*
8	XXIII–	184	84	226	105
13	XXII	185	85	219	100
17	XXI	235	100	215	97
17	"	232	104	207	95
23†	XVII+	94	67	168	89
26†	"	123	78	172	93
27†	"	151	86	185	96
32†	XVI+	120	73	156	81
Avg 20	XIX+	166	85	194	96

* P of cells = .05; of nuclei = .04.
† Metamorphic stasis obtained.

Xenopus. Eight tadpoles were kept in 0.25-0.30% thiourea for 14-35 days. The rate of metamorphosis became progressively slower, and finally metamorphic stasis was achieved in each case, but only after the forelimbs had emerged. The mesencephalic V nucleus cells of experimental and control groups were compared with respect to maximum and average cell sizes, maximum and average nuclear sizes, nucleo-cytoplasmic ratios, and total cell numbers. In only 4 of the 48 comparisons were the anticipated relationships between experimental and control groups unrealized. Of the 6 sets of paired comparisons, only that dealing with cell numbers failed to show significance. Table II shows these relationships.

Fourteen metamorphosed Xenopus were kept in 0.25-0.30% thiourea for 23-32 days (avg 29). As in the larval study, 6 sets of comparisons with 14 matched control animals were made. Maximum and average nuclear sizes were significantly reduced. Maximum cell sizes were slightly lowered (5% level of confidence). The other 3 comparisons failed to show significant differences (Table II). Postmetamorphic age may be an important factor in thyroid hormone control of cell size, since the 7 small juveniles (24-34 mm) showed average cell size reductions 4 times as great

TABLE II. Comparisons of 8 Pairs of *Xenopus* Tadpoles (Upper Group), and of 14 Pairs* of Juvenile Metamorphosed *Xenopus*.

	Max cell size (μ^2)		Avg cell size (μ^2)		Max nuclear size (μ^2)		Avg nuclear size (μ^2)		N/C† ratio		Cell count	
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
	314	337	113	180	85	107	53	68	46	37	151	132
	279	252	138	156	83	96	59	65	43	42	85	92
	246	299	124	172	110	103	63	68	51	39	86	94
	215	316	120	203	88	99	58	68	47	33	108	118
	228	292	119	187	78	100	53	71	44	38	79	127
	246	349	140	192	91	92	64	68	45	36	89	123
	232	369	115	190	76	95	54	67	46	35	116	108
	197	337	82	198	90	97	48	72	58	36	86	99
Avg	245	319	119	185	89	99	57	68	48	37	100	112
p	<.008		<.001		<.03		<.002		<.001		<.25	
	419	493	263	284	104	116	68	79	26	27	94	97
	491	428	339	299	114	119	88	80	26	26	75	83
	357	525	272	318	107	113	76	81	27	25	85	75
	372	421	269	279	103	131	75	80	30	28	90	101
	478	550	303	310	118	124	88	87	29	28	104	105
	484	460	259	260	116	109	74	80	29	30	94	77
	400	392	256	236	108	108	68	68	26	28	77	105
	282	319	157	183	81	109	55	61	35	33	76	106
	320	356	194	174	97	96	61	60	32	34	97	102
	388	424	219	253	94	108	65	73	29	29	90	102
	361	384	213	239	104	104	64	73	30	30	105	97
	345	352	193	208	86	95	59	66	31	32	104	83
	283	280	163	183	76	94	54	62	33	34	106	103
	276	329	188	198	80	91	59	66	31	33	81	79
Avg	375	408	235	244	99	108	68	73	30	30	91	94
p	<.05		<.15		<.01		<.01		<.5		<.25	

* Arranged in order of decreasing body length (65-20 mm).

† $\frac{\text{Avg nuclear size}}{\text{Avg cell size}} \times 100.$

as those shown by the large ones (43-68 mm). Nuclear differences also varied correspondingly.

Discussion. The treatment of *Rana pipiens* larvae with thiourea during the rapid growth phase of the cells of the mesencephalic V nucleus resulted in reduction of the sizes of these cells and their nuclei. This has been even more convincingly shown in the more completely inhibited series with Xenopus larvae, and extended, in part, into the early postmetamorphic period. These results for the larval studies were predicted on the basis of earlier work on the cells of this nucleus(4,6). The realization of the prediction gives added support to the conclusion that level of differentiation of cells of the mesencephalic V nucleus is continuously influenced by the concentration of thyroid hormone in the circulation, in the same sense that cells of the mammalian prostate gland are under the control of androgens(9). A few additional neural cells in amphibians have been shown to respond by either growth or involution(1,2,10) to the addition of thyroid hormone (Mauthner's cell, other cells in the medulla oblongata, lateral motor column cells of the posterior trunk segments of the spinal cord), but thus far only the mesencephalic V nucleus cells have been shown to respond directly to the hormone (see references 3, 4, for discussions and review). That other hormones influence brain cells in other vertebrates is also clearly demonstrated by the influence of hormones upon behavior(11).

Summary. 1. Tadpoles of *Rana pipiens* and *Xenopus laevis* in premetamorphic stages were placed in solutions of thiourea up to 35 days. After 8 days or longer significant slowing of metamorphosis was achieved. Commonly complete arrest of metamorphosis occurred. 2. Compared to values in control animals, the sizes of cells and nuclei of the mesencephalic V nucleus were significantly

reduced, and the nucleo-cytoplasmic ratio was increased, *i.e.*, the changes were toward values characterizing younger larval stages. 3. Similar treatment of juvenile postmetamorphic Xenopus gave comparable but less marked changes in cell and nuclear sizes. The changes were greatest in smaller animals.

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Collagen Digestion by Dog Pancreatic Juice.* (23146)

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Although collagen has long been recognized to be resistant to enzymic hydrolysis, Ssasdikow(1) reported that trypsin-free fractions of pancreatic extract partially degrade collagen. Weak collagenolytic activity was also found in dog pancreatic juice obtained by Pavlov fistula. Recently Ziffren and Hosie(2) reported degradation of collagen by commercial trypsin preparations and pancreatic juice, and postulated the presence of a heat labile enzyme, collagenase, in pancreatic juice. Banga and Balo(3) noted liberation of carbohydrate from collagen during incubation with an enzyme from a pancreatic extract they called collagenmucoproteinase. Certain bacteria, notably *Clostridia,* produce collagenase as an exoenzyme.

Because of the uncertainty of the identity of the enzyme responsible for collagen degradation in dog pancreatic juice, the present study was undertaken in an attempt to define the nature of "collagenase."

Methods. Collagen was prepared from beef achilles tendon by the method of Buechler(4). Electron microscopy† of the preparation revealed that the great majority of particles showed the characteristic structure of native collagen. Crystalline trypsin, chymotrypsin, and soy-bean inhibitor were obtained from Worthington Biochemical Corp., Freehold, N. J. Carboxypeptidase was obtained from Pentex, Inc., Kankakee, Ill. Bacterial collagenase from *Clostridium histolyticum* was generously supplied by Dr. R. E. Kallio of the Department of Bacteriology. Pancreatic juice was obtained from mongrel dogs by the procedures of Ziffren and Hosie(2). Collagen (50 mg) was suspended in 3 ml of pancreatic juice, enzyme solutions, or buffer solutions, each at pH 8.0. After incubation at 37° for 24 hours with occasional shaking, 7 ml of water was added, the suspension was shaken well and centrifuged. The residuum was saved for further study and a portion of supernatant was autoclaved for 3 hours in 6 N HCl at 15 lb/sq. in. An aliquot of the hydrolysate was neutralized to pH 4-5 with 6 N NaOH and the free hydroxyproline was determined by the method of Neuman and Logan(5). Undigested collagen obtained by

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† We are indebted to Dr. J. M. Layton of Department of Pathology for examining the collagen.

TABLE I. Digestion of Collagen by Pancreatic Juice and Proteolytic Enzymes.*

Medium	mg collagen digested: Analysis of supernatant		Analysis of residual collagen	
M/15 phosphate, pH 7.0	1	(3)	3 ± 2	(14)
Trypsin in M/15 phosphate, pH 7 (1.3 mg/cc)	4.3 ± .1	(2)	7.5 ± 1	(2)
Carboxypeptidase (.066 mg/cc)	2.8	(1)		
Pancreatic juice, inactive				
Dog 1			4.1 ± 2	(3)
3	3.6	(1)	6.7	(1)
4	3.1 ± .1	(3)	3.0 ± .7	(2)
Mean	3.3 ± .3		4.6 ± 1.6	
Pancreatic juice, active				
Dog 1	5.7	(1)	9	(1)
2	5.1 ±1.2	(4)	11.6 ± 2.1	(6)
3	4.1	(1)	11.6	(1)
4	4.5 ± .3	(2)	6.9	(1)
Mean	4.9 ± .6		9.8 ± 2	
Pancreatic juice and inhibitor				
Dog 2 active	3.6 ± .8	(3)	8.5 ± 1	(3)
3 inactive	2.9	(1)	8.5	(1)
4 "	3.1 ± .2	(2)	1.5	(1)
4 active	3.2 ± .3	(2)	5.1	(1)
Mean	3.2 ± .2		5.9 ± 2.9	

* Conditions as described in text. Crystalline trypsin inhibitor (2 mg) was added as indicated. The numbers of separate experiments, done in duplicate, are indicated in parentheses.

centrifuging was washed at the centrifuge 3 times with water, discarding the washings. After hydrolysis of undigested collagen with 6 N HCl for 3 hours in the autoclave in the same manner as before, hydroxyproline was determined in an aliquot of hydrolysate diluted 1:100. This method of hydrolysis gave 90% recovery of the hydroxyproline (13.4%) in beef achilles tendon(5). The enzyme results are expressed as mg of collagen dissolved.

Results. The extent of collagen degradation (Table I) as measured by hydroxyproline in the supernatant, was in certain cases much less than the degradation as measured by the difference between collagen added and that recovered after incubation. Mere manipulation and washing made 1.9 mg of the 50 mg of collagen soluble as revealed by recovery of collagen in the residuum; moreover, addition and prompt removal of pancreatic juice followed by the usual washing procedure brought about solubilization of 3.6 mg. Hydrolyzed pancreatic juice contained no significant quantities of free hydroxyproline.

The results obtained by assay of residual collagen were much more variable than those obtained from the supernatant. Both methods of assay, however, demonstrated a clear difference in activity of various pancreatic preparations. For descriptive purposes, these have been called "active" and "inactive." Pancreatic juice from dogs 1 and 3 was initially inactive but became active after repeated freezing and thawing, whereas juice from dog 2 was active immediately following collection. Active pancreatic juice made 6.8 mg or 12% of the collagen soluble by assay of the undigested residue but only 3.9 mg or 6.8% soluble by assay of the supernatant. Inactive pancreatic juice gave about 2.3 mg or 5% digestion with both assay procedures. No difference in carbohydrate content of the supernatant could be detected by using the phenol sulfuric acid method of R. Montgomery.‡ Addition of 0.1 mg of trypsin (sufficient to convert trypsinogen to trypsin) to 20 ml of pancreatic juice from dog 4, caused the preparation to become active. Addition of 2 mg of soybean trypsin inhibitor to active pancreatic juice decreased collagen degradation to the level of inactive pancreatic juice, but had no effect upon inactive preparations. Bacterial collagenase digested collagen rapidly but was not inhibited by soybean trypsin inhibitor.

Trypsin (1.3 mg/3 ml) but not chymotrypsin made about 4.3 mg of collagen soluble under these conditions. Interestingly, carboxypeptidase (0.2 mg/3 ml) also degraded about 2.8 mg of collagen, despite the fact that proline and hydroxyproline peptides are unattacked by this enzyme. Commercial preparations of carboxypeptidase are reported to contain a proteolytic contaminant(6).

Discussion. The study of enzymic degra-

‡ Personal communication.

dation of collagen is complicated by several factors. Apart from the obvious problem of the variable degree of dispersion of the substrate, the chemical constitution of collagen from beef achilles tendon is uncertain. In preparation of collagen the only property employed for its purification is insolubility, a property shared by other structural constituents. Beef achilles tendon collagen is reported to contain about 4% of elastin(7), which is subject to destruction by pancreatic elastase. Approximately 5% of our collagen preparation was insoluble after heating in water for 3 hours at 100°, conditions which convert collagen to gelatin. However, elastin contains only about 1.8% of hydroxyproline; therefore collagen breakdown measured as hydroxyproline is not significantly affected by elastin breakdown. In addition, denatured collagen, which is distinguished from native collagen by differences in appearance by electron microscopy, but which cannot be measured quantitatively, may be more susceptible to enzymic destruction. Neuman and Tytell(8) have emphasized the importance of employing native collagen for collagenase assay. By electron microscopy, our preparation, which was predominately native collagen, contained small amounts of denatured collagen. In the present experiments a decision cannot be made as to whether native or denatured collagen was being degraded.

A major portion of collagen digestion appears to be due to trypsin. This conclusion is based on activation of fresh pancreatic juice by small amounts of trypsin and by inhibition of collagen digestion by activated pancreatic juice in the presence of specific soybean inhibitor.

Indeed the differences observed between assays of hydroxyproline in supernatant solution and in washed residue suggest that tryptic digestion may be manifested not only by dissolution of collagen but also by weakening of the structure so that it becomes more soluble upon subsequent washing. Hide collagen pretreated with trypsin subsequently becomes more soluble in warm water(9).

Ziffren and Hosie(2) reported that dog pancreatic juice digested large quantities of collagen. Their methods differed from ours in 3 respects. (1) The residual collagen, separated by filtration, was transferred to test tubes for subsequent hydrolysis. Losses by manipulation could cause considerable error. (2) The residual collagen was hydrolyzed for 30 minutes with 0.5 N HCl at 15 lb/sq. in. pressure. Under these conditions we found only 5-7% of total hydroxyproline was liberated; even after 3 hours heating only 60% of hydroxyproline was liberated. (3) The colorimetric standard previously employed was not purified hydroxyproline but was a weighed portion of collagen which was also hydrolyzed with 0.5 N HCl for 30 minutes at 15 lb pressure. Moreover a buffer-collagen control was not run with each experiment. One or more of these possible sources of error may have magnified the extent of collagen digestion by pancreatic juice reported previously.

The extent of collagen digestion by pancreatic enzymes other than trypsin is so slight that it is difficult to ascertain the nature of the causative enzyme. Possibly carboxypeptidase, pankrin(10), prolinase, or elastase singly or in combination are responsible. Until methods for the preparation of pure native collagen are developed, or specific inhibitors for those other enzymes are found, attempts to define this small digestion do not appear fruitful. In our opinion, it is unnecessary to ascribe the small amount of collagen degradation caused by inactive or inhibited pancreatic juice to a specific pancreatic collagenase.

Summary. Degradation of collagen after one day incubation with pancreatic juice or various enzymes was studied. About 6% was degraded by trypsin or by pancreatic juice containing active trypsin. Collagen degradation was much less when incubated with fresh inactive pancreatic juice or pancreatic juice containing trypsin inhibitor. The extensive digestion of collagen previously reported could not be confirmed.

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Chemotherapeutic Activity of Combinations of Sulfisoxazole and Oleandomycin. (23147)

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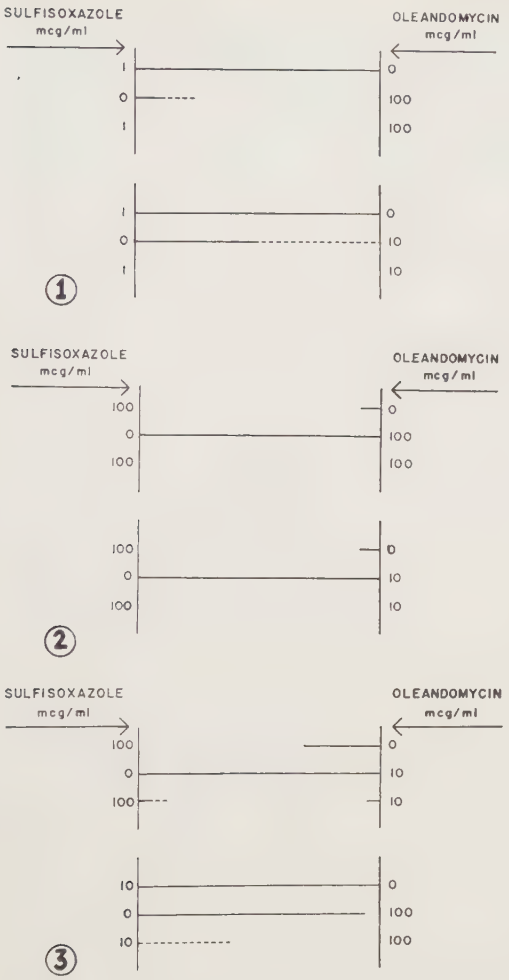
The combined effect of antibacterial agents generally demonstrated by an increased but also occasionally decreased bacteriostatic effect *in vitro* has comparatively rarely been reproduced in animal experiments. This report is concerned with a comparison of combined activity of a sulfonamide and an antibiotic *in vitro* and *in vivo*. The agents used were: (a) sulfisoxazole (3,4-dimethyl-5-sulfanilamido-isoxazole)* which has been described(1) as a substance of low toxicity and marked though not always optimal(2) chemotherapeutic activity *in vitro* and in experimental infections with Gram positive and Gram negative organisms, and (b) oleandomycin, an antibiotic which has been shown by Sobin *et al.*(3) as well as by Fust *et al.*(4) to exhibit *in vitro* and in experimental animals an activity range similar to that of penicillin and erythromycin. Clinically the effect of the antibiotic is particularly marked against Gram positive microorganisms(5).

Materials and methods. The origin of the strains(1) as well as the technics of the broth dilution tests(1) and gradient plates(6) have been described elsewhere. Mueller-Hinton medium(7) was used in all experiments which employed the gradient plate. The methods for the *in vivo* experiments are the same as those given by Schnitzer *et al.*(1) except that the Giorgio strain of *Staphylococcus aureus* (obtained through the courtesy of Dr. R. M. McCune, Jr.) which is of low sensitivity to sulfonamides was used. The infective dose of this organism was 0.5 ml intravenously of a saline suspension containing 250,000,000 cells. Both *in vitro* in the broth dilution method and *in vivo,* sulfisoxazole and oleandomycin were tested alone as well as in the following combinations: (a) a fixed relation of 5 parts sulfisoxazole combined with 1 part oleandomycin, (b) sulfisoxazole in the presence of a constant inactive concentration of oleandomycin, and (c) oleandomycin in the presence of a constant, inactive concentration of sulfisoxazole. *In vitro* the combination of sulfisoxazole and oleandomycin was also tested in the proportion of 1:1.

Results. Activity *in vitro.* A. Broth dilution method: In the case of *Streptococcus hemolyticus* 4 and *Pneumococcus pneumoniae* 6301, sulfisoxazole showed comparatively low activity (25-50 mg/ml) while oleandomycin was highly effective (0.25-0.5 μg/ml). With *Escherichia coli* J and *Eberthella typhi* F the range of activity was 3.9 to 15.6 μg/ml for sulfisoxazole and 31.2-62.5 μg/ml for oleandomycin. The inhibiting dose against *Staphylococcus aureus* 209 was 3.9 μg/ml for sulfisoxazole and 0.031 μg/ml for oleandomycin. In every case, the combination gave the effect of the more potent component.

B. Gradient plate technic. The results of experiments with *Staphylococcus aureus* 209, *Escherichia coli* J and *Eberthella typhi* F,

* Gantrisin®.



Graphic demonstration of activity on gradient plates of sulfisoxazole, oleandomycin and combinations of the two substances against:

FIG. 1. *Staphylococcus aureus* 209.
FIG. 2. *Escherichia coli* J.
FIG. 3. *Eberthella typhi* F.

carried out on the gradient plate(6) are graphically depicted in Fig. 1, 2, and 3 respectively. In these figures the arrow indicates the gradient of the substance in a particular layer and the numeral gives the maximum concentration of the substance incorporated. The heavy line between two numerals indicates normal heavy growth, the broken line, light bacterial growth and the absence of a line between two numerals, no growth. Fig. 1, 2 and 3 contain examples of combinations which exhibited an effect superior to that of the single drugs in *Staph. aureus* 209, *E. coli* J and *E. typhi* F respectively. With *E. typhi* F (Fig. 3) at a concentration of 100 μg/ml sulfisoxazole and 10 μg/ml oleandomycin the results show not only the superior effect of the combination but also growth at the highest point of concentration of sulfisoxazole. This growth could be a function of a too heavy inoculum but could also indicate possible interference of minute amounts of oleandomycin with the high concentration of sulfisoxazole under the present experimental conditions. Such an occurrence is feasible since the drug combination balance showing increased or decreased effect is often critical and extremely close in values for a combination of agents.

Chemotherapeutic activity *in vivo*. The activity of the combination of a fixed relation of 5 parts sulfisoxazole and 1 part oleandomycin as compared to the single constituents against the 3 Gram positive (streptococci, pneumococci and staphylococci) and the one Gram negative (salmonella) organisms are given in Table I. From the PD_{50} values (50% protective dose), which were calculated according to the method of Reed and Muench(8) on the basis of animals surviving the 21-day observation period, it can be seen that in the case of streptococci, pneumococci and staphylococci the values obtained for the constituents of the combination are less than that of each substance alone. This can be interpreted as indicating a superior effect of the 5 to 1 combination.

In the case of *Salmonella schottmuelleri* where oleandomycin is completely inactive, the experimental results only show the effect of the active component, sulfisoxazole.

When sulfisoxazole in the presence of a constant inactive concentration of oleandomycin was tested against *Strep. hemolyticus* 4 and *Pn. pneumoniae* 6301, the activity of sulfisoxazole in the combination was essentially the same as that observed when the substance was used alone. The same type of effect was observed against *Pn. pneumoniae* 6301 and *S. schottmuelleri* with oleandomycin in the presence of a constant inactive concentration of sulfisoxazole. However, in an

TABLE I. The *In Vivo* Antibacterial Activity of the Combination of Sulfisoxazole and Oleandomycin in a Fixed 5 to 1 Ratio as Compared with the Single Constituents.

mg/kg/os		*Strep. hemolyticus* 4		*Pn. pneumoniae* 6301		*Staph. aureus* (Giorgio)		*S. schottmuelleri*	
Sulfisoxazole	Oleandomycin	% survival	mg/kg PD_{50}	% survival	mg/kg PD_{50}	% survival	mg/kg PD_{50}	% survival	mg/kg PD_{50}
2000				90	1500	10	>2000		
1000				0					
250								90	
125		80						50	100.6
62.5		40	80.8					50	
31.2		20						10	
	2000							10	>2000
	200			90		90			
	100			50	79.5	50	87.7		
	50	90		20		20			
	25	50	23.3						
	12.5	10							
500	100			70		70			
250	50			50	283.3 + 56.7	30	264.5 + 52.9	90	
125	25	100		10		40		60	92.8 + 18.6
62.5	12.5	60	50.1 + 10			30		40	
31.2	6.25	20						0	
Effect of combination		enhanced		enhanced		enhanced		none	

infection with *S. schottmuelleri,* sulfisoxazole combined with a constant inactive dose of oleandomycin (1000 mg/kg) was more than twice as active (59.1 mg/kg) as it was when given alone (149.7 mg/kg).

Discussion. The experimental evidence has shown that under certain conditions both *in vitro* and *in vivo* the combination of the sulfonamide, sulfisoxazole, and the antibiotic, oleandomycin, exerts an effect superior to that of the single constituents. If one attempts to define the conditions which govern the demonstration of the combination effect, it is evident that the serial dilution tests *in vitro* as well as the combination with inactive doses of either agent *in vivo* failed to show increased activity. The more delicate balance maintained in the gradient plates where the abrupt differences of arbitrary dilution steps are avoided demonstrates, however, the enhanced effects of sulfisoxazole and oleandomycin as long as one member exerted at least a partial bacteriostatic activity. The experiments in animals indicate that the fixed relation of doses as given by the 5 to 1 proportion of sulfisoxazole to oleandomycin represents a suitable mixture. It causes a moderate, usually less than a 2-fold, reduction of oleandomycin. The required amount of sulfisoxazole can be considerably lower dependent on the sensitivity of the organism. In infections caused by organisms insensitive to one of the 2 compounds, *e.g. S. schottmuelleri* and oleandomycin, activity of the effective constituent usually prevails. However, even under these conditions an enhancing effect has been observed.

Since these observations do not seem to fit into any of the more or less well defined concepts of synergism, we prefer the expressions "enhanced" or "supplementary" effect to describe the results obtained in the present experimental study. The supplementation of sulfisoxazole consists in the accentuation of its Gram positive quota through combination with oleandomycin. The latter substance is supplemented by the marked influence of sulfisoxazole on Gram negative organisms. Thus, one of the aims of combination therapy, namely the broadening of the antibacterial spectrum, has been accomplished in the sulfisoxazole-oleandomycin mixture.

Furthermore, recent studies on the high frequency of resistant staphylococci, even to penicillin, seem to make it desirable to include substances of potent effect against these or-

ganisms and without cross-resistance to the majority of other clinically useful antibiotics. Oleandomycin is an agent which, with the exception of cross-resistance to members of the erythromycin group, displays these properties.

Summary (1) *In vitro* under the specific conditions of the gradient plate technic, combinations of sulfisoxazole and oleandomycin in specific ratios show an activity superior to that observed with the single constituents. (2) Supplemental activity was also observed *in vivo* in the streptococcal, staphylococcal and pneumococcal infections when the ratio of the combination of sulfisoxazole to oleandomycin was 5 to 1. (3) The same type of effect was also observed in *Salmonella schottmuelleri* infection of mice when activity of sulfisoxazole was determined in a constant, inactive amount of oleandomycin.

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Inactivation of Foot-and-Mouth Disease Virus by pH and Temperature Changes and by Formaldehyde. (23148)

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Methods of tissue culture for production of foot-and-mouth disease virus, type A, and for assay of the virus and its neutralizing antibodies were reported by Bachrach, Hess, and Callis(1). These methods have now been employed for measurements of stability of tissue culture virus under variable environmental conditions. The present report is concerned with rates of inactivation of virus by the action of hydrogen ion, heat, and formaldehyde.

Materials and methods. Virus. Foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), was produced in Roux flask cultures of bovine kidney tissue(1). For preparation of the cultures, cut fragments of bovine kidney tissue were dispersed by incubation in 0.25% trypsin-buffer solution according to Youngner's modification(2) of the Dulbecco and Vogt(3) procedure for monkey kidney tissue. The liberated cells were decanted through 3 layers of gauze, centrifuged at low speed, washed twice in phosphate-buffered saline(3), and suspended at a 1:200 dilution in culture fluid. The fluid consisted of Hanks' salt solution containing 2% bovine serum and 0.5% lactalbumin hydrolyzate (Nutritional Biochemicals Corp.). Penicillin G, streptomycin sulfate, and phenol red were included at final concentrations of 100 units/ml, 0.1 mg/ml, and 0.005%, respectively. Roux flasks were each seeded with 75 ml of the cellular suspension and incubated at 37°C. Fluid changes were made after 3 and 6 days' incubation. By the second fluid change the cultures consisted of confluent outgrowths of epithelial and fibroblastic cells. The flasks containing the cultures were then inoculated with 4 ml of infective fluid from the preceding tissue culture passage and, after

being plugged with cotton, were incubated at 37°C. Infected culture fluids harvested 18 to 24 hours later contained as high as $10^{7.5}$ Reed and Muench(4) 50% infectivity doses per milliliter for bovine kidney cultures (TC ID_{50}/ml). These fluids after adjustment to pH 7.5 with dilute acid or alkali were sealed in ampules and stored at –40°C. When needed for experimental work the frozen virus was thawed to 4°C and clarified by centrifugation at 1,500 rpm for 10 minutes. *Kidney cell cultures in tubes were used for assaying virus.* In their preparation 0.4-ml volumes of cellular suspension prepared as described above were pipetted into culture tubes, 16 x 150 mm. The tubes were stoppered tightly and laid as close to horizontal as possible without wetting the stoppers. Incubation was carried out at 37°C, and fluid changes were made after 3, 6, and 8 days. The resulting outgrowths were suitable for use in the bioassay of virus between the sixth and the tenth day of growth. *For bioassay of virus,* serial 10-fold dilutions of the infectious samples to be tested were made in fresh culture fluid. Eight-tenth-ml volumes of the dilutions were inoculated into assay cultures, 5 cultures per dilution. After 48 hours' incubation, distribution of recognizable cytopathic changes was recorded, and TC ID_{50} values calculated. Cultures not showing these changes within 48 hours very rarely become infected upon further incubation.

Results. pH stability. The stability of FMDV-A119 was determined at pH values ranging from 2 to 10. Portions of thawed and clarified virus fluid were diluted 1:10 in cold veronal-acetate buffers of Michaelis(5) at pH 5, 6, 6.5, 7, 7.5, 8, 9, and 10. Additional virus samples were diluted in acidified saline at pH 2, 3, and 4. These acidified and buffered virus solutions were held in stoppered flasks at 4°C and were sampled periodically for virus assay. To avoid injuring cells of the assay cultures by adverse concentrations of hydrogen ions, the samples taken for assay were readjusted to pH 7.5 with dilute HCl or NaOH solutions as required. These adjustments were carried out with stirring to minimize development of high, local concentrations of the acid or base. In most cases buffer capacities were sufficient to hold pH values to within 0.05 of a unit of the nominal values over a period of several weeks. For continuous control, the pH value of each stored sample was determined at each sampling, and if found to deviate by more than 0.1 unit from the nominal value, a readjustment was made with dilute HCl or NaOH.

The inactivation curves for the 8 preparations in veronal-acetate buffer are shown in Fig. 1. The virus was most stable at pH 7 or 7.5, where its infectivity decreased only slightly over the entire 5-week period of observation. At pH 8 and 9 viral inactivations occurred at rates of approximately 90% per 3- and 1-week period, respectively. For samples stored at pH values 6.5 and 10, there was a 90% reduction in infectivity every 14 hours, and for samples at 5 and 6 a similar reduction was observed in less than 1 minute. Under conditions of this experiment the rates of inactivation of virus in the 3 acidified solutions at pH 2, 3, and 4 were too rapid to be measured.

Refinements in technics were required to determine more precisely the rapid rates of inactivation of acidified virus. A procedure was developed in which virus was instantaneously adjusted to the required pH value without subjecting it to conditions more acid than that desired. After being acidified for as short as 4 seconds, the virus was instantly neutralized without exposure to adverse alkaline conditions. A description of the technic, all steps of which were carried out at 4°C, follows. One part of the virus suspension was introduced carefully into the bottom of a test tube without contaminating the walls higher up in the tube. Nine parts of veronal-acetate buffer properly adjusted with respect to pH were poured into the virus, simultaneously agitating the tube to insure rapid mixing. To stop the reaction, an equal volume of neutralizing veronal-acetate buffer was poured into the virus-buffer mixture. The neutralizing buffer was adjusted beforehand to that alkalinity required to bring the acidified virus to pH 7.5. Virus was tested in this manner for its stability at pH 3, 4, 5, and 6.

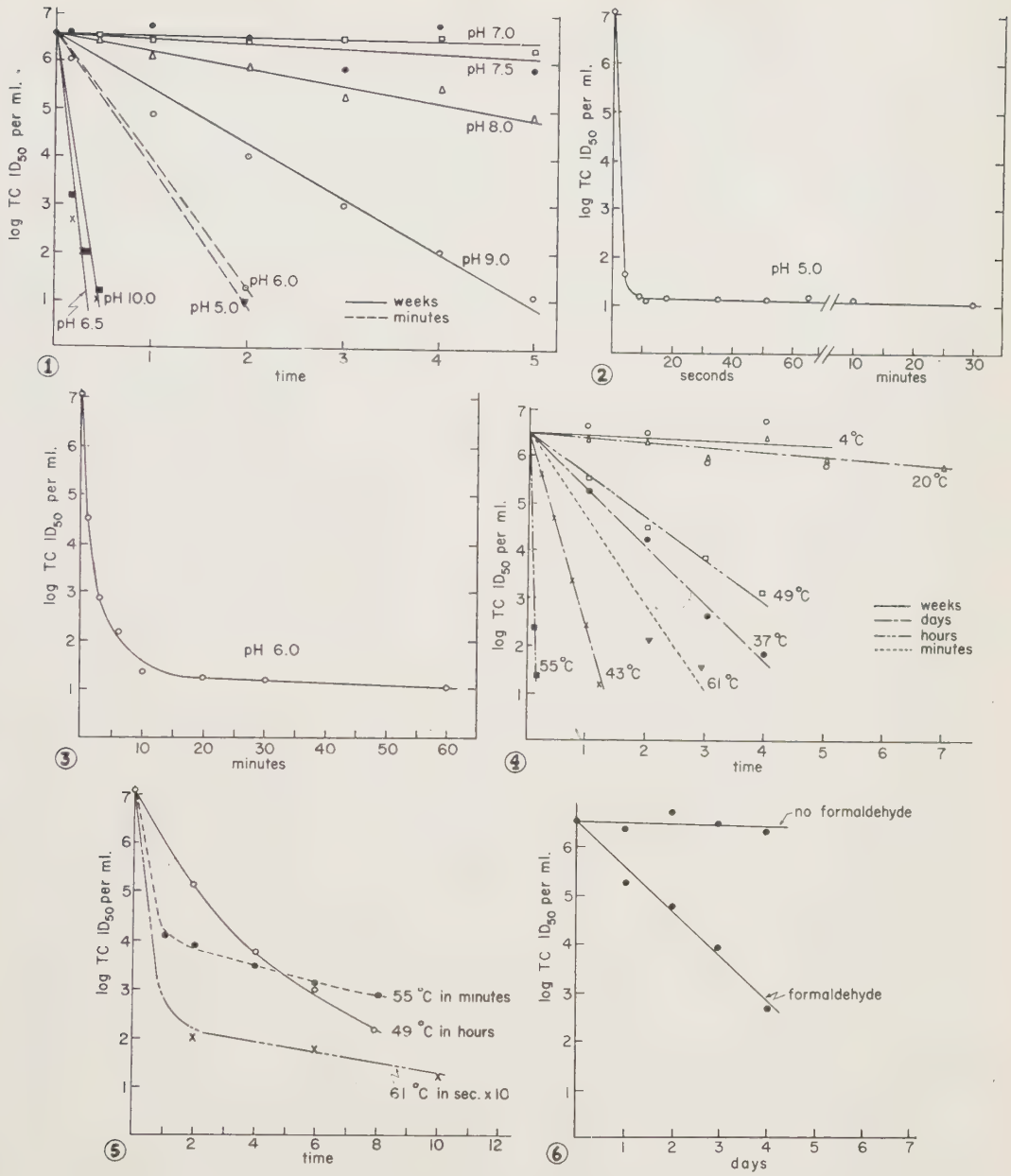

FIG. 1. pH stability of tissue culture foot-and-mouth disease virus, type A, at 4°C.

FIG. 2. Inactivation at pH 5.0 of tissue culture foot-and-mouth disease virus, type A, at 4°C. One-millionth part of the population remains infective for at least 30 min.

FIG. 3. Inactivation at pH 6.0 of tissue culture foot-and-mouth disease virus, type A, at 4°C, showing that about one-millionth of the population has a much greater stability than the rest.

FIG. 4. Thermal stability of tissue culture foot-and-mouth disease virus, type A, at pH 7.5.

FIG. 5. Inactivation at 49°, 55° and 61°C of tissue culture foot-and-mouth disease virus, type A, showing that at low survival levels the virus has increased heat stability.

FIG. 6. Inactivation of tissue culture foot-and-mouth disease virus, type A, by formaldehyde at a concentration of 0.009%.

It was found that veronal-acetate buffers adjusted to each of these pH levels brought the virus precisely to the nominal values. The pH values of the buffers required for neutralization were 11.60, 11.28, 8.70, and 8.15, respectively. Zero time values of infectivity were determined by titration of the original stock virus properly diluted in veronal-acetate buffer at pH 7.5.

Fig. 2 shows that virus was inactivated at pH 5 more rapidly than formerly depicted (Fig. 1), and in addition approximately one-millionth of the original virus population was resistant to further inactivation for at least 30 minutes. Fig. 3 shows that initial rate of inactivation at pH 6 was like that inferred in Fig. 1, but similar to the finding at pH 5, approximately one-millionth of the virus population was persistent to inactivation for a minimum of 60 minutes. This persistent fraction was present even in virus fluids which had been clarified at 10,000 rpm for 30 minutes before adjustment to pH 6. Inactivation of virus at pH 3 or 4 was so rapid that only one-millionth part of the infectivity remained after exposure for 10 seconds; after 45 seconds all infectivity detectable by tissue culture methods had disappeared.

Thermal inactivation rates of FMDV-A119 were determined from 4° to 61°C. A thawed and clarified virus suspension was diluted 1:10 in veronal-acetate buffer at pH 7.5. Portions of this suspension were placed in small Erlenmeyer flasks and brought rapidly to the temperatures shown in Fig. 4. At regular intervals samples were removed from each flask for virus assay. From the curves (Fig. 4) it was calculated that the time intervals required for thermal inactivation of 90% of the virus existing at any time were as follows: 0.5 minute at 61°, 2 minutes at 55°, 1 hour at 49°, 7 hours at 43°, 21 hours at 37°, 11 days at 20°, and 18 weeks at 4°C.

Additional experiments were devised which would permit a more exact study of the kinetics of rapid inactivation occurring at higher temperatures, namely, 49°, 55°, and 61°C. A procedure was employed in which virus suspensions at 20°C were raised instantly to the desired temperature and, after known intervals of exposure, were cooled instantly to 10°C. To accomplish this, one part of buffered virus at 20°C was mixed with 9 parts of veronal-acetate buffer held in a water bath at one-half degree higher than the temperature desired. An instantaneous adjustment of temperature was thereby effected. After a minimum interval of 15 seconds the reaction was stopped by diluting the heated virus sample with an equal volume of cold buffer. Typical results of several experiments are shown in Fig. 5. The results at 49°C were in close agreement with those found previously (Fig. 4) except for a slightly increased thermal stability of a small portion of the virus. At 55° and 61°C the inactivation rates were at least tenfold greater than those depicted earlier (Fig. 4) until survivals of 0.001 and 0.00001 were reached, respectively. (Survival refers to ratio of concentration of virus before and after exposure.) The virus fractions remaining after these survival levels were reached had greatly increased heat stabilities.

Formaldehyde inactivation. Inactivation of FMDV-A119 by formaldehyde at 4°C and pH 8 was determined. Frozen infected tissue culture fluid was thawed to 4°C, clarified, and then adjusted to pH 8 by dilution (1:10) in veronal-acetate buffer. Untreated portions of this fluid and portions treated with formaldehyde at a concentration of 0.009% were stored at 4°C and sampled daily for virus assay. Fig. 6 shows that formaldehyde-treated virus was inactivated at a rate of 90% per day. A similar result was found for virus which was adjusted to pH 8 with dilute NaOH, instead of buffer, prior to treatment with formaldehyde. At the time of formaldehyde experiments only the results involving short exposure at various pH levels were known (*i.e.*, Fig. 1, results through 3 days), and these did not indicate significant stability differences in the range of pH 7 to 8. Moreover, in the untreated control of the formaldehyde experiment (Fig. 6), there was little or no loss of infectivity due to exposure at pH 8 alone.

Discussion. The lability of FMDV-A119 of tissue culture origin under slightly acid

conditions is of particular interest. This behavior is in marked contrast to that of poliovirus, which is completely stable at a pH as low as 1.5 for at least 24 hours at 4°C(6). Such extreme differences in resistance of 2 animal viruses must reflect comparable differences in physical and chemical composition. The one-millionth part of the infective FMDV that resists inactivation at pH 5 and 6 disappears on lowering the pH to 4. Since virus populations clarified at 10,000 rpm for 30 minutes before acidification to pH 6 still contained a pH resistant fraction, the residual infectivity was probably not due to particles protected by aggregation. Nor were specific ions of the buffer involved since the controls in buffer at pH 7.5 did not lose infectivity. The residual infectivity was probably not due to reactivation of virus upon neutralization since there were no points of inflection in the pH curves. Studies are now in progress to determine if pH persistence is related to environmental conditions or genetic differences.

The importance of maintaining FMDV between pH 7 and 7.5 has already been observed in virus growth experiments carried out in Roux flask cultures of bovine kidney tissue. The pH in tightly stoppered cultures drops from 7.3 to 6.8 within a few hours after infection, with a concomitant decrease in the yield of virus. Once detected, this situation was corrected by providing for the escape of respiratory carbon dioxide by closing the flasks with cotton plugs instead of rubber stoppers at time of inoculation with virus.

The resistance of a small fraction of the virus to rapid inactivation at 55° and 61°C (Fig. 5) raises questions concerning the homogeneity of the population. Experiments are now under way to determine the basis of heat resistance and if it is related to pH persistence.

Activation energies for loss of infectivity have been derived from an Arrhenius plot(7) of thermal data (Fig. 7). The inactivation process can be described by first-order kinetics. The reaction rate constants were derived from the data of Fig. 4 except for the temperatures 49°, 55°, and 61°C, where the

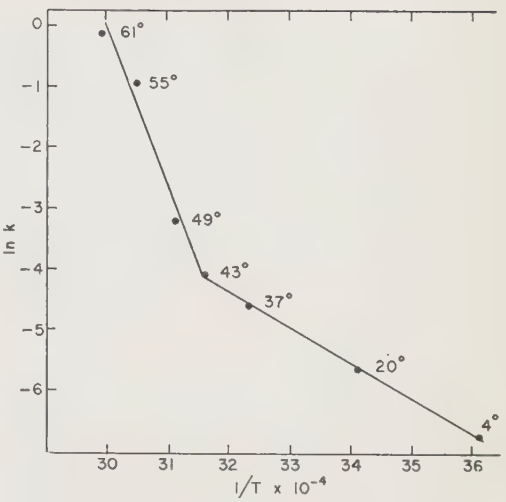

FIG. 7. Arrhenius plot of the thermal data for inactivation of foot-and-mouth disease virus. Logarithm of the rate constants for inactivation, *k*, *vs* the reciprocal of the absolute temperature, 1/T.

rates were determined from the initial slopes of the curves in Fig. 5. The Arrhenius plot gave 2 straight lines of different slope joining at about 43°C. Activation energies calculated from the slopes below and above 43°C were 27,200 and 120,600 calories per mole of FMDV, respectively. This result indicates that loss of infectivity by thermal action may have proceeded by 2 different processes. Speculating on the nature of such processes, Lauffer and Price(8) produced evidence at least with tobacco mosaic virus that high temperatures inactivated virus by denaturing the protein moiety faster than unknown thermal effects at lower temperatures.

Under conditions of temperature and pH approximately optimal for preservation of infectivity, FMDV was inactivated by formaldehyde according to first-order kinetics at a rate of about 90% per day (*i.e.*, close to that caused by exposure at 37°C alone). No part of the virus population appeared to possess above-normal resistance to formaldehyde. However, because of a lack of suitable technics, data for inactivations approaching zero infectivity were not experimentally obtainable. Thus, the time required for total inactivation of virus by formaldehyde is not necessarily determined by extrapolation of the slope in Fig. 6 to zero infectivity.

Summary. 1) Rates of inactivation of tissue-culture-derived, foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), at various pH levels and temperatures and by formaldehyde were determined. Ranges of pH and temperature investigated were 2.0 through 10.0 and 4°C through 61°C, respectively; formaldehyde was employed at 0.009%. The results are interpretable by first-order kinetics. However, at pH 5 and 6 and also at 55° and 61°C small fractions of the virus population had much lower first-order inactivation rates than the bulk of the virus. Possibilities concerning the nature of the fractions with higher resistance are discussed. 2) Rates of inactivation at various pH levels were determined at 4°C. Below pH 4 the virus was totally destroyed within a few seconds. At pH 5 and 6 infectivity was lost at a rate of about 90% per second and minute, respectively, until only one-millionth of the virus remained. This residual virus was very stable to further inactivation. At pH 6.5 and 10, 90% of the virus was inactivated every 14 hours. The virus showed marked stability only at pH 7 and 7.5, losing little infectivity during a 5-week period. At pH 8 and 9, a 90% reduction of infectivity occurred within a 3- and a 1-week period, respectively. 3) Rates of thermal inactivation were determined at pH 7.5. The time intervals required for the inactivation of 90% of the virus existing at any time were as follows: 18 weeks at 4°; 11 days at 20°; 21 hours at 37°; 7 hours at 43°; 1 hour at 49°; 20 seconds at 55° to a survival of 0.001, 7 minutes thereafter; and 3 seconds at 61°C to a survival of 0.00001, 11 minutes thereafter. Activation energies calculated for loss of infectivity below and above 43°C were 27,200 and 120,600 calories per mole of FMDV, respectively. 4) Virus treated with formaldehyde at a concentration of 0.009% was inactivated at a rate of 90% per day of storage at 4°C.

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Weekly Variations in Serum Cholesterol Levels of Monkeys. (23149)

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Since the original reports of Peterson(1,2) on the hypocholesterolemic effect of soy sterols on cholesterol-fed chickens, many papers have appeared concerning the effect of soy sterols and b-sitosterol in lowering serum cholesterol levels in cholesterol-fed rabbits (3) and cholesterol-eating humans(4,5,6). The latter observation has not been confirmed in all instances(7). It has been suggested that the soy sterols exert their effect by interfering with the absorption of dietary cholesterol(4,8). In experiments using cholesterol-C^{14} interference was inferred by Chaikoff(9) and Kritchevsky(10) but not by Rosenman (11). The question of what influence soy sterols have on reabsorption of cholesterol has not been answered. As an effort in this direction we fed b-sitosterol to monkeys on a diet that was essentially cholesterol-free. Feedings were carried out daily for 6 weeks, with the animals being observed for two weeks before and 6 weeks after the feeding period. We observed no inordinate changes in the serum cholesterol levels either before or after the

TABLE I. Serum Cholesterol Levels (mg %) in Monkeys before, during and after Sitosterol Administration.

Monkey No.	Weeks 1	2*	3	4	5	6	7	8†	9	10	11	12	13	14
A	141	114	128	140	127	111	140	111	lost	145	110	107	131	118
B	132	161	175	186	181	132	149	130	144	143	127	144	155	119
C	122	137	115	130	117	153	141	137	121	214	124	132	131	124
D	86	90	95	128	104	95	109	118	111	131	148	127	145	117
E	146	159	184	157	129	137	168	127	143	257	137	137	152	149
F	lost	113	122	134	95	84	107	96	98	92	127	98	123	98

* Animals on 1 g sitosterol daily after this bleeding.
† Animals off sitosterol after this bleeding.

feeding experiment, but did see swings in the weekly cholesterol levels which resemble those reported in humans by Wilkinson(12), and which have also been noted by Fahrenbach.*

Methods. Six male Cynomolgus monkeys weighing 4-6 lbs were used. Each animal was bled weekly and the serum cholesterol levels determined in duplicate by the method of Trinder(13). This was done for 14 weeks, as follows: for 2 weeks before sitosterol feeding, during the 6 weeks of feeding, and for 6 weeks thereafter. Feedings of sitosterol were daily, by stomach tube, and one gram was delivered in each case. The basic diet of the monkeys consisted of Monkey Wafers (Dietrich and Gambrill, Inc., Frederick, Md.) containing soy flour, wheat germ, soy bean oil meal, dry skim milk, alfalfa meal, sucrose, bone meal, salt and vitamins. The wafers were augmented with fruit.

Results. The protocol for the feeding of sitosterol to human beings involves amounts ranging from 9 to 24 g daily(14) given in divided doses before each meal. In the monkey experiment, the gram of sitosterol was administered just before the animal's single daily feeding. This dosage for a 4-6 lb monkey compares well with that fed to patients.

TABLE II. Average Serum Cholesterol Levels (mg %) in Monkeys during and after Sitosterol Feeding.

Monkey No.	Sitosterol feeding*	Range	Post-sitosterol*	Range
A	126	111-140	122	107-145
B	159	130-186	139	119-155
C	132	115-153	141	121-214
D	108	95-128	130	111-148
E	150	127-184	165	137-257
F	106	84-134	106	92-127

* Period of observation—6 wk.

* Unpublished data.

Two things became evident from the data presented in Tables I and II. First, sitosterol feeding to normocholesterolemic animals on a diet devoid of cholesterol does not change serum cholesterol levels appreciably. This finding could support the belief that the mode of action of sitosterol is by interference with the absorption of dietary cholesterol. The second observation is that normal variations in cholesterol levels from week to week are so great as to obscure changes that might be brought about by the sitosterol. Wilkinson (12) has argued that in view of these wide swings, great care must be taken in establishing base levels for serum cholesterol in experiments designed to measure any outside effects. More data, covering longer time intervals, must be accumulated to evaluate overall cholesterol variations.

Summary. Six monkeys maintained on a diet essentially free of cholesterol were fed 1 g sitosterol daily for 6 weeks, and serum cholesterol levels were assayed before, during and after the feeding period. The data present no clear-cut evidence that the sitosterol lowered the level of serum cholesterol. The animals all exhibited wide swings in weekly cholesterol levels not related to sitosterol intake.

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Insulinase-Inhibitory Action of Metabolic Derivatives of L-Tryptophan.* (23150)

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A decrease in blood sugar concentration of normal rats is produced by a number of metabolic derivatives of L-tryptophan administered by stomach tube(1). Since these compounds are not effective in the severely diabetic alloxanized rat, the hypoglycemic response produced in the normal animal cannot be due to either a decrease in availability of some hyperglycemic agent or to an acute hepatotoxic action and inhibition of enzyme systems involved in glycogenolysis. Insulin dependency of the hypoglycemic response, however, may result from either an increase in secretion of insulin or a decrease in destruction of endogenous insulin consequent to an inhibition of insulinase. Accordingly, the influence of various metabolic products of L-tryptophan on the activity of insulinase *in vitro* and *in vivo* was determined.

Methods. Extracts of livers from fed, male Carworth rats were prepared as described previously(2). The incubation mixture consisted of 1 ml of extract plus 1 ml of Sörensen's M/15 phosphate buffer(3) containing 20 units of a mixture of I^{131} labeled and unlabeled insulin as well as various concentrations of the metabolic products of L-tryptophan. As a control, the same mixture was incubated without the L-tryptophan derivatives. At the end of 30 minutes of incubation at 37°C and pH 7.8, 2 ml of 10% trichloracetic acid was added to the incubation mixture and the quantity of insulin degraded was computed as previously described(2) from the percentage of total radioactivity which appeared in the trichloracetic acid filtrate. The metabolic products of L-tryptophan† used, were indole-3-acetic acid, anthranilic acid, kynurenic acid, picolinic acid, quinolinic acid, nicotinic acid, nicotinamide, nicotinuric acid, 5-hydroxytryptophan, 5-hydroxytryptamine creatinine sulfate, 5-hydroxyindoleacetic acid, indole and skatole. These compounds were dissolved or suspended in 0.5% sodium bicarbonate and adjusted to pH 7.8. To determine the effect of the various compounds on insulinase activity of the intact animal, groups of 10 fed male mice were given a subcutaneous injection of 0.004 mM of the L-tryptophan derivatives in 0.05 ml/gram body weight. Immediately thereafter the mice were given a subcutaneous injection of 1 ml of 5.5% solution of glucose. One hour later,

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one group of mice was given an intraperitoneal injection of 0.4 unit insulin in 0.02 ml acidified water/gram body weight and another subcutaneous injection of 1 ml of the 5.5% glucose solution. The insulin consisted of a mixture of crystalline zinc insulin and tracer quantities of I^{131} labeled insulin. Groups of 10 mice which were given sodium bicarbonate instead of the L-tryptophan derivatives served as controls. All mice were sacrificed one hour after injection of the insulin. The preparation of the animals and determination of the quantity of insulin destroyed/hour/100 g of mouse was the same as that described previously(4).

Results. The data on the effect of various metabolic products of L-tryptophan on destruction of insulin by fresh extracts of rat liver are summarized in Table I. All but 2 of the compounds exerted some degree of insulinase inhibition. Whereas nicotinic acid produced no effect, nicotinamide produced an increase in rate of destruction of insulin by the fresh extracts.

The action of some of the compounds on destruction of insulin by intact mice is depicted in Fig. 1 which also illustrates the action of equivalent dosages of the same compounds by mouth on blood sugar concentration of rats. Those compounds which produced a decrease in blood sugar concentration of the rat produced also a statistically significant ($P<0.001$) inhibition of the destruction of exogenous insulin by intact mice. Quinolinic acid which had no significant influence on blood sugar concentration of rats and nicotinamide which induced a marked hypoglycemia, produced a significant increase in insulinase activity of mice. The action of nicotinic acid, indole and skatole on destruction of insulin by intact mice was not established since these compounds were toxic in the quantities employed.

TABLE I. Effect of Metabolic Products of L-Tryptophan on Inhibition of Destruction of Insulin by Extracts of Rat Livers.

Compound	Inhibition, mean ± % .1 M	.05 M	.025 M
Kynurenic acid	63.5 ± 1.4	35.5 ± 1.3	39.0 ± 1.6
Indole-3-acetic acid	60.6 ± 1.6	56.9 ± 2.0	26.9 ± 5.2
5-Hydroxyindole acetic acid	57.7 ± 3.2	65.0 ± 3.2	32.5 ± 2.7
L-Tryptophan	56.6 ± 1.3	57.0 ± 1.1	29.2 ± 1.6
5-Hydroxytryptophan	56.4 ± 2.0	57.8 ± 1.3	18.8 ± 1.6
Nicotinuric acid	56.1 ± 1.2	49.7 ± 1.6	15.6 ± 2.3
Anthranilic acid	45.8 ± 1.5	36.5 ± .3	2.4 ± 1.3
5-Hydroxytryptamine	43.8 ± 3.7	13.6 ± 2.4	8.8 ± 1.1
Picolinic acid	42.5 ± 4.3	38.4 ± .6	8.8 ± 2.0
Quinolinic acid	35.0 ± 6.6	38.4 ± .6	19.3 ± .6
Indole	25.6 ± 1.7	23.3 ± .9	13.5 ± 2.9
Skatole	12.5 ± 5.8	16.6 ± 1.2	8.6 ± 4.3
Nicotinic acid	1.3 ± 1.0		5.1 ± 3.1
Nicotinamide			−50.4 ± 1.3

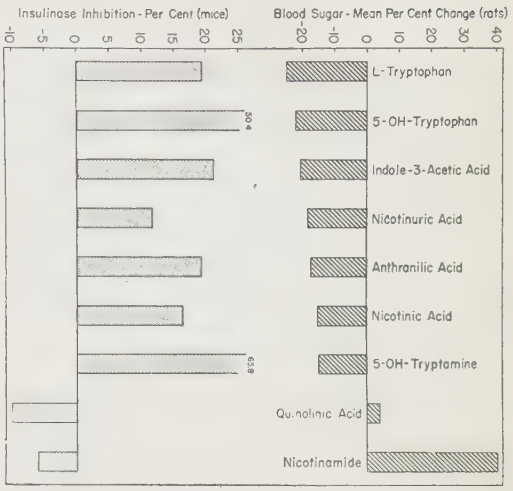

FIG. 1. Effect of metabolic products of L-tryptophan on destruction of insulin by intact mice. The areas represent mean % change in blood sugar conc. of 10 rats during a 5 hr interval after dosage of 4 mM of the compound/kilo body wt. The areas represent % of inhibition of insulinase as computed from difference in rates of insulin destruction by groups of intact mice given sodium bicarbonate and of those given 4 mM of the designated compound/kilo body wt.

It is evident that most of the compounds which exert an insulinase-inhibitory activity *in vitro* exert a similar effect *in vivo*. A discrepancy exists, however, between the action of nicotinic and quinolinic acids *in vitro* and *in vivo*. The positive correlation between effect of the various compounds on blood sugar concentration of rats and effect on rate of destruction of endogenous insulin by mice suggests that the *in vivo* effects are of greater

physiological significance than are the *in vitro* effects.

The data reported herein support the hypothesis that the hypoglycemic action of L-tryptophan and of some of its metabolic derivatives is related to a decrease in the rate of destruction of endogenous insulin. The precise mechanism involved remains unknown.

Conclusions. Metabolic derivatives of L-tryptophan which produce a hypoglycemic response in rats are effective also as inhibitors of the destruction of insulin by fresh extracts of rat livers and by intact mice.

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Action of Ouabain upon Normal and Hypodynamic Myocardium.* (23151)

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The positive inotropic action of cardiac glycosides upon hypodynamic isolated mammalian cardiac muscle has been demonstrated many times. The papillary muscle of the cat has been used most frequently, and the usual experimental procedure has been to stimulate the muscle, suspended in a phosphate medium containing glucose, over a period of hours until it becomes hypodynamic and to then add the drug(1,2). Isolated papillary muscles become hypodynamic within a few hours in phosphate media, in contrast to their stability in bicarbonate media(2). It has been claimed that the typical increase in force of contraction upon addition of non-toxic concentrations of cardiac glycosides does not occur in myocardium which is not hypodynamic, *e.g.,* in bicarbonate medium(2) or in phosphate medium when the drug is added prior to the development of the hypodynamic state(3). We have investigated this matter in guinea pig myocardium.

Methods. Isolated, electrically stimulated strips, prepared from the right ventricular wall of the guinea pig (150-200 g males) heart in a manner similar to that described previously for the rat(4), were used. The basic medium employed had the following composition (m*M*): NaCl 154, KCl 5.6, and $CaCl_2$ 3.2. The bicarbonate medium contained in addition 5 m*M* $NaHCO_3$ and was gassed with 1% CO_2-99% O_2 mixture (final pH 7.4). The phosphate medium contained 1 m*M* sodium phosphate buffer in place of bicarbonate and was gassed with 100% O_2 (final pH 7.4). Both media contained 0.1% glucose. The strips were stimulated at 37°C at a rate of 100/minute at a constant resting tension of 750 mg. The absolute values of the "initial force" were of the same order of magnitude in the two media (approximately 100 mg).

Results. Phosphate medium. Fig. 1 shows the results of typical experiments in which ouabain was added to a hypodynamic preparation (A) and to one in which the force of contraction had not fallen appreciably from the initial value (B). Magnitude and duration of the positive inotropic actions were similar by comparison with the control values.

Bicarbonate medium. Fig. 2 shows the effect of the same concentration of ouabain upon a strip in bicarbonate medium. A positive inotropic action occurred in this typical experiment which persisted above the control value for many hours. The great stability of the muscle in the bicarbonate medium, in contrast to its behavior in phosphate medium, is evident in this figure.

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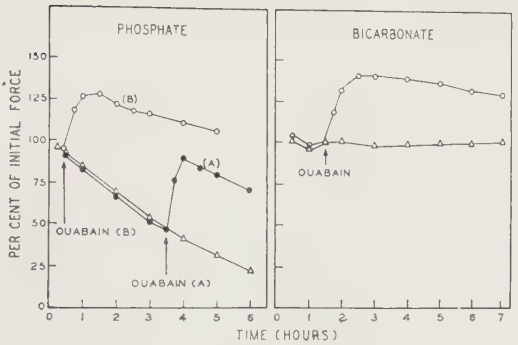

FIG. 1 (left). Effect of ouabain (1:6,000,000) upon force of contraction of ventricular strips in phosphate-buffered medium. –●–●– drug added to hypodynamic strip (A); –○–○– drug added soon after setting up strip (B); –△–△– control strip (no drug added). "Initial force" defined as force extrapolated to zero time.

FIG. 2 (right). Effect of ouabain (1:6,000,000) upon force of contraction of ventricular strips in bicarbonate-buffered medium. –○–○– drug added as indicated; –△–△– control strip (no drug added). "Initial force" defined as force after equilibration period of approximately 1.5 hr.

The concentration of ouabain used above (1:6,000,000) was considerably less than that required to produce a toxic effect upon the muscle. A concentration of 1:1,000,000 led consistently to the appearance of spontaneous contractions, the rate of which was independent of the frequency of the stimulator, and to a marked increase in resting tension. Such toxic effects of high concentrations of cardiac glycosides upon papillary muscles have been noted previously(1). A concentration of 1:10,000,000 produced a positive inotropic action in bicarbonate medium of approximately one-half that shown in Fig. 2.

Discussion. The view that a hypodynamic state must be produced in isolated mammalian myocardium in order to demonstrate a positive inotropic action of cardiac glycosides has resulted in the use of unphysiological (*e.g.*, phosphate-buffered) media in order to induce this state(1,2). The unphysiological nature of phosphate in comparison with bicarbonate media is evident from the steady decrease in developed tension which occurs in the former and from the failure of glucose to restore the force of contraction normally after prolonged contraction in substrate-free phosphate medium(4). Our finding that the use of such media is not necessary in order to demonstrate the typical action of ouabain implies that the system(s) in guinea pig myocardium affected by the cardiac glycosides with resultant increased force of contraction are present and affected as readily in "normal" as in hypodynamic isolated myocardium. It might be noted that a positive inotropic response to cardiac glycosides occurred also in dog heart *in situ* in the absence of evidence of failure (5).

Summary. The action of ouabain upon force of contraction of guinea pig ventricular strips in phosphate and bicarbonate media was studied. A positive inotropic response occurred in both "fresh" and hypodynamic strips in phosphate medium, and also in bicarbonate medium.

We wish to thank the Allan Hancock Foundation for the use of facilities during this investigation.

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Anti-Heparin Activity of Erythrocyte Hemolysate.* (23152)

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Quick and his co-workers(1) discovered clotting activity resembling platelet thromboplastic activity in erythrocyte hemolysate. DeVries and his associates(2) suspended in saline the residue of a sedimented erythrocyte hemolysate previously extracted with water. This material contained platelet thromboplastic but not anti-heparin activity. In contrast, we have found that Quick's whole hemolysate preparation possesses anti-heparin activity comparable to that of concentrated platelet suspensions.

Reagents. Erythrocyte hemolysate was made by Quick's technic(3) of freezing and thawing washed, platelet-free erythrocytes in physiological saline. Undiluted hemolysate consisted of hemolyzed cells suspended in a volume of saline equal to the original plasma volume. *Intact red blood cells* were the same cells not disrupted by freezing and thawing. *Platelets* were obtained in a concentration of about 11,000,000/cu mm by differential centrifugation and suspension after washing, in a diluting fluid consisting of 3 parts of physiological saline, 1 part of 0.025 M sodium citrate and 1 part of sodium diethyl barbiturate (veronal) buffer. *Cephalin,* an acetone insoluble, ether soluble fraction of human brain, was made as previously described(4). *Saline brain extract thromboplastin* and *veronal buffer* were prepared by Owren's technic(5), and *oxalated, platelet-poor plasma* by high-speed centrifugation of fresh plasma handled with "silicone technic." A bovine *thrombin solution* of 100 units/ml, was made by dissolving the contents of one vial of Parke-Davis Topical Thrombin in a mixture of 25 ml of saline and 25 ml of glycerine. This stock preparation retained full activity for weeks at 4°C. Dilute solutions were prepared in buffer with "silicone technic" and used within 30 minutes. Heparin (Abbott, Heparin Injection 1%) was diluted in saline and added *in vitro* to plasma. Plasma from patients receiving heparin (Upjohn, Heparin sodium, 100 mg/ml) was also examined. The reagents and technic of the prothrombin-proconvertin (P. and P.) assay(6) are described elsewhere.

Results. 1. *Evidence that hemolysate can abolish the anti-thrombic activity of heparin.* Table I summarizes results obtained by mixing 0.4 ml of platelet-poor plasma containing 0.044 unit of heparin/ml with 0.2 ml of material being examined for anti-heparin activity. After 3 minutes at 37°C, 0.2 ml of dilute (5 units/ml), warmed thrombin solution was added and the clotting time recorded. The thrombin potency was checked at the end of the experiment by clotting plasma containing saline instead of heparin. This Table shows that erythrocyte hemolysate, platelets and saline brain extract effectively neutralize heparin. Intact red cells have no, and "cephalin" only weak, anti-heparin activity. Wolf had previously noted(7) that lipid brain extracts could not inactivate heparin.

Table II lists clotting times obtained when 0.2 ml of different concentrations of hemolysate, platelets and "cephalin" were mixed with 0.4 ml of platelet-poor plasma containing 0.037 unit of heparin/ml, and the mixture clotted with 0.2 ml of thrombin as described above. The final concentration of test material in the clotting mixture, therefore, was one-fourth of the concentration given in the Table. As Table II illustrates, an initial hemolysate concentration of 1/25 (final concentration of 1/100) exhibited some anti-heparin activity. Intravascular hemolysis of sufficient intensity to raise the plasma hemoglobin level to 150 mg% would produce approximately this concentration of red cell products *in vivo*. Table II also shows that platelets possess definite anti-heparin activity at their normal circulating concentra-

* This study was supported by grant from the Los Angeles County Heart Assn.

TABLE I. Effect of Various Materials upon Anti-Thrombic Activity of Heparin.

	Test material	Thrombin time (sec.)
Normal plasma containing heparin &	Dil. fluid	No clot
	Intact RBC	Sl. clot 720 sec.
	Cephalin (1/50)	87, 243
	Hemolysate	30, 28
	Platelets	38, 40
	Thrombopl.	42, 42
Saline	Dil. fluid	29, 28

tion (150-300,000/cu mm of blood by the Rees-Ecker method used). "Cephalin" was ineffective at any concentration studied.

Note that thrombin time of mixture of heparinized plasma and undiluted hemolysate, 23 seconds, was shorter than that of the control without heparin or hemolysate, 33 seconds. Therefore, the thrombin time of a mixture of hemolysate and plasma was compared with that of a mixture of saline and plasma without added heparin.

Table III summarizes the results obtained when first 0.2 ml of hemolysate, and then saline, were added to 0.4 ml of oxalated and

TABLE II. Effect of Various Concentrations of Hemolysate, Platelets and "Cephalin" on Thrombin Time of Heparinized Plasma.

Material	Initial conc.	Thrombin time (sec.)
Hemolysate	Undil.	23
	1/ 5	38
	1/10	55
	1/25	135
	1/50	no clot
	x1000	
Platelets	10,860/mm³	34
	5,430	29
	2,715	29
	1,358	33
	679	31
	340	36
	170	60
	85	188
	42	499
"Cephalin"	Undil. (1.1 g %)	no clot
	1/ 10	535
	1/ 25	227
	1/ 50	no clot
	1/100	" "

Thrombin time of control mixture of 0.4 ml of "non-heparinized" plasma and 0.2 ml of diluting fluid was 33 sec.

TABLE III. Effect of Hemolysate upon Thrombin Time in the Absence of Heparin.

Type of plasma	Added material	Thrombin time (sec.)
Oxalated	Saline	64, 68
	Hemolysate	46, 46
Citrated	Saline	53, 52
	Hemolysate	43, 44

citrated plasma. The mixture was clotted with 0.2 ml of a weaker thrombin solution (1 unit/ml) to magnify differences. As this Table reveals, hemolysate was capable of shortening the thrombin time in the absence of added heparin. This implies that while hemolysate shortens the thrombin time of heparinized plasma primarily because of its anti-heparin activity, it also exerts a secondary, more direct effect upon the rate of the thrombin-fibrinogen reaction. A similar property of platelet extracts has been reported(8).

2. *Evidence that hemolysate abolishes heparin interference with prothrombin consumption.* Heparin not only inactivates preformed thrombin but interferes with conversion of prothrombin to thrombin. Therefore, if hemolysate contains anti-heparin activity, it should increase the rate of prothrombin consumption in clotted blood containing heparin. To test this, 2 ml samples of blood from patients receiving heparin were added to each of 4 small tubes. One tube contained no added material, the other 3 contained, respectively, 0.2 ml of buffer, of "cephalin," and of hemolysate. Each tube stood for 1 hour at 37°C after clotting before 0.2 ml of 0.1 M sodium citrate was added to halt prothrombin conversion. The residual serum prothrombin was then estimated by the P. and P. method of Owren and Aas(6).

These data are given in Table IV. The prolonged whole blood clotting times confirm the presence of heparin in the blood at the time of the test. The normal plasma P. and P. values illustrate the reliability of this assay in the presence of heparin. The failure of either buffer or "cephalin" to improve the impaired prothrombin consumption of the heparinized blood again illustrates their lack of anti-heparin activity. The very low P.

TABLE IV. Residual P. and P. Levels in Sera from Heparinized Blood Clotted with Buffer, "Cephalin" and Hemolysate.

Clotting time (13 min. normal), min.	P. and P. levels (%)				
	Plasma	Serum			
		Material added			
		Nothing	Buffer	"Cephalin"	Hemolysate
20	100	39	51	55	<4
21	86	33	33	35	"
20	74	44	31	36	"
17	82	27	15	28	<2

and P. levels obtained with hemolysate demonstrate strikingly its anti-heparin activity.

Discussion. Quick's erythrocyte hemolysate resembles platelet suspensions in possessing both platelet thromboplastic like activity and anti-heparin activity, and in directly shortening the time of the thrombin-fibrinogen reaction. Thus, hemolysate differs from commonly used lipid extract platelet substitutes, such as "cephalin," which possess only platelet thromboplastic like activity. DeVries (2) work indicates that thromboplastic activity of hemolysate can be separated from its other clotting properties.

Hemolysate can be made easily and stored frozen indefinitely. Therefore it is a convenient reagent to test for heparinemia. Our procedure is to add 0.2 ml of a standardized dilute thrombin solution that will clot normal plasma in about 20 seconds to a mixture of 0.4 ml of the plasma to be tested and 0.2 ml of saline. A normal thrombin time rules out heparinemia. If a long thrombin time is obtained, or if no clot forms, the test is repeated substituting 0.2 ml of hemolysate for the saline. If this corrects the long thrombin time, heparinemia should be strongly suspected.

If hemolysate fails to shorten a prolonged thrombin time, heparinemia is not its cause. However, if clotting fails to occur either with saline or with hemolysate, a further step is necessary. Failure to clot could mean that so much heparin is present that enough remains after hemolysate neutralization to inactivate the dilute thrombin used. Therefore, the test must be repeated using a thrombin solution strong enough to clot the mixture containing saline. Then, failure to obtain a much shorter time with hemolysate will definitely eliminate heparinemia.

The use of hemolysate also permits an evaluation of the plasma thromboplastic factors in heparinized blood. This becomes necessary, for example, when bleeding occurs in a patient receiving heparin in amounts that should not be excessive. If, in this circumstance, hemolysate corrects abnormal prothrombin consumption of the heparinized blood (see again Table IV) a co-existing deficiency of anti-hemophilic globulin (AHG), plasma thromboplastic component (PTC) or plasma thromboplastin antecedent (PTA) would be most unlikely.

Summary. 1. Erythrocyte hemolysate possesses anti-heparin activity that can correct the prolonged thrombin time and impaired prothrombin consumption of heparinized blood. This makes erythrocyte hemolysate a useful reagent to detect heparinemia and to permit the evaluation of plasma clotting factor activities in heparinized blood. 2. Erythrocyte hemolysate also produces some shortening of the thrombin time of plasma not containing added heparin.

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Evidence on Inheritance of Muscular Dystrophy in an Inbred Strain of Mice Using Ovarian Transplantation.* (23153)

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An inherited muscular dystrophy which occurs in inbred strain 129 mice has been reported by Michelson, Russell, and Harman (1). The disease was described as a primary myopathy characterized by ataxia, atrophy, and paralysis, and is similar to the Duchenne type of human muscular dystrophy. Evidence was presented which suggested that the abnormality occurs in animals homozygous for a recessive autosomal mutation to be called *dystrophia muscularis* and designated by the symbol *dy*. Muscular dystrophies with a unit hereditary basis are known in other species. There are 3 commonly occurring types of this disease in humans, all hereditary(2,3,4,5). The Duchenne type is evidently transmitted by a sex-linked recessive gene. The facioscapulohumeral type appears to be inherited by an autosomal dominant factor, although the possibility that sometimes it may be sex-limited (to females) has not been ruled out. Limb-girdle muscular dystrophy is probably inherited as an autosomal recessive character, although in some pedigrees the gene appears to have a dominant effect. A muscular abnormality in New Hampshire fowl which grossly and microscopically resembles muscular dystrophies in man has been reported by Asmundson and Julian(6). Judging from data derived from various types of matings, the abnormal birds are homozygous for an autosomal recessive gene (*am*). The potentialities of hereditary muscular dystrophy in mice as a research tool in the study of muscular disease made it desirable to consider procedures which would facilitate production of affected animals in quantity. Russell and Douglas(7), using the technic of ovarian transplantation developed by Robertson(8), demonstrated that offspring can be obtained from transplanted embryonic ovaries, and suggested that this technic could be a valuable tool in experimental zoology. In particular they mentioned that ovaries of lethal genotypes could, when grafted to normal hosts, make possible a direct genetic test of the lethal type itself. This method has since been successfully used to obtain large numbers of offspring by Russell and Hurst(9), Little *et al.*(10), and Stevens(11). The less economical procedures of artificial insemination and ova transplantation were considered impractical for use in obtaining large numbers of offspring. Russell and Hurst(9) worked out a method of using "stock 129" (now called strain 129) mice as donors of ovarian grafts, and as hosts F_1 hybrids with strain 129. This system exploits the fact that strain 129 mice are maintained with forced heterozygosity for a coat color gene, and has the distinct advantage of enabling the investigator to identify each offspring positively as derived from the graft or the reconstituted host ovary. Although the breeding data presented for mouse dystrophy by Michelson *et al.*(1) strongly suggested unit autosomal recessive transmission, 2 characteristics of the entity make it difficult to obtain evidence directly supporting this hypothesis. One is the impossibility of recognizing carriers phenotypically, which led to the incorporation into the pedigree of many matings with at least one non-carrier parent. The second difficulty is the short life-span and physical disability of the dystrophic mice, which prevents successful mating. The evidence presented by these authors was the incidence (25/117, or 21%) in F_2 descendants from ovaries of 2 dystrophic females grafted homoiotopically into normal hosts.

In this paper we present further evidence based on matings of animals of identified genotype which confirms the hypothesis that

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dystrophia muscularis is inherited as a unit recessive. Furthermore, an example is given which illustrates that the technic of ovarian transplantation is practical in obtaining large numbers of offspring from ovaries grafted from females of extremely low fecundity.

Materials and methods. Strain 129 mice have been inbred for more than 20 generations by mating brothers and sisters heterozygous for a pair of alleles which determine whether the animals are albinos or pigmented. The colors resulting from recombinations of these alleles are: white ($c^a c^a$), chinchilla ($c^{ch} c^{ch}$), and a hue intermediate between these ($c^a c^{ch}$). In most cases, chinchilla females with muscular dystrophy (*dy dy*) were used as donors of ovarian grafts. The hosts were black agouti females obtained from mating albino strain 129 with DBA mice. The technic of ovarian transplantation is essentially that described by Robertson(8), although a few modifications have been introduced. Each operation was performed with clean instruments, including Swiss-made watchmaker's forceps with fine points, iridectomy and dissecting scissors. The animals were anaesthetized with ether. The donor was sacrificed and ovaries removed from the ovarian capsules, bisected, and kept moist within the body cavity of the donor until the host was prepared to receive the graft. A mid-dorsal incision approximately 1 cm in length was made through the skin at the level of the ovaries. The incision was drawn laterad and a second incision, through the body wall, was made just ventral to the dorsal muscle mass, and the ovary was exteriorized by pulling out the fat pad attached to the ovarian capsule. A small cut was made in the ovarian capsule so it could be peeled back to expose the ovary. The ovary was removed by pinching it off at the hilus, and the graft of ½ an ovary was inserted into the empty capsule. It is important to remove as much of the ovary as possible so that reconstitution will not occur. The encapsulated graft was then inserted into the body cavity. The skin incision was drawn to the other side of the animal and the remaining host ovary with its capsule and oviduct was removed. The skin incision was closed by a wound clip. It has been established that when donors approximately 6 weeks old are used, grafts of one half of an ovary perform, reproductively, better than grafts of a whole ovary or ¼ of an ovary (Stevens, unpublished). The hybrid females, bearing grafts from dystrophic donors were mated with normal (*Dy Dy*) or heterozygous (*Dy dy*) males. The normal males were obtained from a colony of strain 129 mice known to be free from dystrophic individuals. Two classes of offspring, unaffected (*Dy dy*) and dystrophic (*dy dy*) resulted. The normal heterozygous offspring were mated *inter se*. All heterozygous animals used in breeding experiments were offspring derived from *dy dy* ovarian grafts.

Results. Reproductive performance of animals bearing ovarian grafts. The reproductive performance to date (2/7/57) of animals bearing ovarian grafts from donors with muscular dystrophy is summarized in Table I.

Since the grafts consisted of halves of ovaries, approximately 75 dystrophic donors were used. Of 294 females with ovarian grafts, 205 (69%) have produced 2348 graft offspring which have survived until weaning age. Only 39 of these females which have produced exclusively graft offspring have been observed throughout their reproductive period, the remaining ones being currently under observation. The average number of litters from the 39 mice was 5.1 per female and the average litter size at birth was 4.1.

TABLE I. Reproductive Performance of Normal Females Carrying Ovarian Grafts from Donors with Muscular Dystrophy.

No. operations	No. females producing offspring from: Graft ovary	Host ovary	Mixed	No. offspring born	No. offspring weaned:* Graft origin	Host origin
294	150	4	55	3234	2348	162

* Some mice younger than weaning-age were used for experimental purposes.

TABLE II. Classification of Offspring Derived from Matings of *Dy Dy* Females Carrying *dy dy* Ovarian Grafts and from Matings of Heterozygous Animals (*Dy dy*) *inter se*.*

Parent genotype	No. weaned	No. weaned with muscular dystrophy	%
dy dy ovarian graft × *Dy Dy* ♂	815	0	0
dy dy ovarian graft × *Dy dy* ♂	1413	626	44
Dy dy ♀ × *Dy dy* ♂	2211	422	19

* Individuals from host or mixed litters or litters from which animals younger than weaning age were removed for experimental purposes are not included in this table.

The number of litters per female ranged from 1 to 9 and the size of the litters varied from 1 to 11. These data make it apparent that the technic of ovarian transplantation is practical for use in direct genetic studies using gonads from genotypically lethal mice. In addition, the technic is shown to be satisfactory in producing large numbers of offspring.

Genetic segregation of Dy and dy alleles. The results of breeding F_1 hybrid females bearing ovarian grafts of the genotype *dy dy* with normal (*Dy Dy*) and heterozygous (*Dy dy*) males are presented in Table II along with the results of breeding heterozygous animals (*Dy dy*) *inter se*. All *Dy dy* individuals used as parents were offspring of *dy dy* grafted ovaries. Although mice with muscular dystrophy can usually be distinguished from their normal littermates at 2 weeks of age, we have included in our results only animals which survived to weaning age when in all cases the disease can be positively identified.

When females with ovarian grafts from dystrophic donors were mated with *Dy Dy* males, all of the offspring were unaffected. When these females were mated with heterozygous males, slightly less than half (44%) were affected with the disease. *Inter se* matings (*Dy dy* × *Dy dy*) of offspring of transplanted ovaries yielded 19% affected young. Male and female offspring were affected in approximately the same numbers. The proportions of dystrophic animals resulting from these 3 types of matings approach those that would be expected if the affected animals were homozygous for an autosomal recessive gene.

Discussion. An example is provided here of use of the technic of ovarian transplantation to facilitate genetic study of an abnormality resulting in early death and drastically reduced fecundity. Russell and Douglas(7) have suggested the use of this procedure to permit direct breeding tests of lethal genotypes.

To make use of the technic of ovarian transplantation, it is necessary that donors and hosts be histocompatible. Furthermore, the donor-host combination must be such that offspring derived from the graft can be distinguished from those derived from possible reconstituted host ovarian tissue. In some cases, the latter requirement is easily met by use of a genetic marker. It is particularly fortunate that the *dy* mutation occurred in the inbred strain 129 which is maintained heterozygous for a coat color gene.

On the basis of breeding data of heterozygous offspring (*Dy dy*) of transplanted ovaries, Michelson *et al.*(1) suggested that *dystrophia muscularis* is caused by homozygosity for a single mutant autosomal recessive gene. Our larger body of data completely confirms this hypothesis. The proportional deficiency of observed vs. expected dystrophics is approximately the same in matings between heterozygotes (19% instead of 25%) as in matings between heterozygous males and animals with homozygous ovarian grafts (44% instead of 50%). One possible explanation for this deficiency is differential mortality of genetically dystrophic animals before they could be classified phenotypically. An alternative explanation was presented by Michelson *et al.*(1) who suggested that the expression of the gene may be masked occasionally by its environment. It is possible that both differential mortality and incomplete penetrance are involved.

The genotype of offspring of transplanted *dy dy* ovaries can always be accurately assigned, since all must contain at least one *dy* gene. These animals provide a reliable supply of known *Dy dy* heterozygotes for use in production matings. The positive identifica-

tion of parental genotypes with respect to the *Dy* and *dy* alleles provides a sound basis for expecting certain ratios of normal:affected offspring. This is particularly useful in the search for early differences between normal and affected individuals.

The method of ovarian transplantation has the added advantage of putting to use the germ cells of affected homozygotes which would otherwise be unavailable for reproductive purposes. Compare the incidence of dystrophic animals in the 1951-55 breeding colony of strain 129 mice with that in the 1956 colony maintained with the help of ovarian transplantation. Between 1951-55, in spite of considerable effort toward selection of matings between heterozygotes, the incidence of dystrophics was 6% (177/2950)(1). In 1956, the combined incidence of dystrophics among offspring of the 3 types of matings listed in Table II was 1048/4439, or 24%. The use of animals with ovarian grafts has greatly facilitated the production of dystrophic mice in quantity and has permitted direct genetic tests which have provided definitive evidence that *dystrophia muscularis* in strain 129 mice is inherited on a unit autosomal recessive basis.

Summary. 1. Critical evidence is given which supports the hypothesis that *dystrophia muscularis* is inherited in strain 129 mice on a unit autosomal recessive basis. 2. Offspring were obtained from ovaries of dystrophic females transplanted to histocompatible normal hosts. The incidence of dystrophy in a large F_2 population descended from these grafted ovaries is 19%, and in a large backcross population 44%. 3. Approximately 70% of the animals bearing ovarian grafts yielded graft offspring. The technic of ovarian transplantation, as modified from that of Robertson is described. 4. Using offspring of known genotype derived from transplanted ovaries, it has been possible to provide a reliable and extensive supply of dystrophic animals and normal littermates for research use.

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Fe^{59} Assessment of Erythropoiesis in Mice Following Injections of Anemic Rabbit Plasma Extracts.* (23154)

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Carnot and DeFlandre(1) postulated the existence of an erythropoietic stimulating factor in plasma of rabbits. Since then much evidence(2-5) has been published favoring a humoral control of red cell production. However, the effectiveness of an erythropoietic factor in deproteinized plasma extract from rabbits made anemic with phenylhydrazine in producing erythropoietic stimulation as judged by Fe^{59} uptake in red cells has not been

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shown to date in mice.

The following report describes specifically the use of the Fe^{59} uptake method to determine effectiveness of deproteinized anemic rabbit plasma extracts in stimulating erythropoiesis in Swiss mice.

Materials and methods. (a) Male Swiss mice (Hauschka ICR Swiss) aged 8-10 weeks and ranging in weight from 18-35 g were employed in all the experiments described below. They were maintained on a diet consisting of Derwood-Morris mouse pellets. (b) White New Zealand rabbits were given 1 cc of 2.5% phenylhydrazine subcutaneously daily until hemoglobin was less than 5 g. They were then bled and the plasma prepared as described previously(2). Control plasma was obtained from normal non-treated rabbits. The deproteinized plasma extract administered was concentrated to ⅓ the original volume of plasma. Swiss mice received subcutaneously 2 cc of rabbit deproteinized concentrated anemic or normal extract for 3 successive days. On the fourth day, 1 μc of Fe^{59} in ½ cc saline was injected into the jugular vein and 24 hours thereafter the mice were bled from the dorsal aorta. The radioactivity in the sample was measured in a Nancy Wood well-type scintillation counter. Radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Calculation of the Fe^{59} uptake into the red cells was carried out as previously described(6). Precaution was taken after each experiment to run hematocrits. Any animal with excessively low hematocrit was not included in our results since animals with low hematocrits had tended to run higher iron uptakes. In this study hypertransfusions with mouse blood were used to test radioiron uptakes as a means of reflecting erythropoiesis in the mouse. This will be described separately along with the results for convenience. The Fe^{59} was injected and calculated in the same manner as indicated above.

TABLE I. Percentage Incorporation of a Single Intravenous Injection of Fe^{59}* into Circulating Erythrocytes of Normal Male Swiss Mice.

Hr	No. of mice	Avg uptake, % and range	S.D.	Avg Hct., % and range
24	23	22.8 (7.9-34.7)	± 2.4	41 (28-48)
48	27	30.3 (10.3-53.6)	± 8.7	42.7 (40-48)
72	27	31.3 (12.2-49.6)	± 2.9	42.3 (37-48)

* Fe^{59} uptake at varying intervals after single inj.

TABLE II. Depression of Red Cell Iron Uptake* by Repeated Blood Transfusions in Normal Male Swiss Mice.

Hr	No. of mice	Avg uptake, % and range	S.D.	Avg Hct., % and range
24	11	2.6 (.3- 4.5)	± 1.2	46.8 (43-51)
48	11	4.8 (2.5- 7.7)	± 1.4	45.3 (42-50)
72	12	9.5 (5.3-12.5)	± 2.0	42.5 (40-45)

* Uptake at varying intervals after a single intrav. inj. of Fe^{59}.

Results. Fe^{59} uptake values are shown in Table I along with hematocrits. The percentage incorporation of a single intravenous injection of Fe^{59} into circulating erythrocytes of normal Swiss mice is expressed as a function of time. The radioiron uptake values in a 24, 48 and 72 hour period were respectively 22.8% ± 2.4, 30.3% ± 8.7, and 31.3% ± 2.9.

To test the validity of radioiron in determining red cell production in mice the following experiment was carried out. It is known that when the product of a tissue or cell is supplied exogenously the producing tissues will often atrophy. A group of normal inbred Swiss mice were given intraperitoneally repeated transfusions of ½ cc of whole blood from Swiss mice for 5 days. On the 5th day capillary hematocrits and red cell counts were done on a representative group of transfused and control mice. The mean of red cell counts in controls was 11.2 million per cu mm and in transfused mice 14.7 million per cu mm. The capillary hematocrits in the controls averaged 41.5% and in the transfused mice 47% in one group and 64% in another. On the sixth day following the last transfusion the mice were given a single intravenous injection of Fe^{59} and were bled 24, 48 and 72 hours thereafter (Tables II and III). Tables II and III show the marked failure of the transfused mouse to utilize the radioiron in red cell production. This is particularly observable in the 24-hour values shown in Tables II and III. However, the 72-hour values show a greater percent uptake than in

TABLE III. Depression of Red Cell Iron Uptake* by Repeated Blood Transfusions in Normal Male Swiss Mice.

	No. of mice	Avg wt (g) and range	Avg uptake, % and range	S.D.	Avg Hct., % and range
Hypertransfused	7	23.4 (19.5-26.0)	.9 (.4- 2.4)†	± .75	62.6 (47-79)
Non-hypertransfused	7	24.4 (18.0-27.0)	17.9 (9.7-30.3)†	± 8.2	41.6 (37-45)

* At 24 hr. † t = 5.4791 P = 1%.

the 24 and 48-hour values in Table II.

The results obtained with deproteinized anemic and normal rabbit plasma extracts are presented in Tables IV and V. In Table IV the first lot of concentrated anemic plasma gave an averaged Fe^{59} incorporation in 24 hours of 38.3% ± 8.01 and the concentrated normal plasma values were 30.8% ± 8.6. In this experiment the differences in mean Fe^{59} incorporation between mice receiving anemic and normal rabbit plasma were significant at a level between 2 and 5% (t – 2.259). The groups in Table V are similar except an untreated control group was added. The second lot of concentrated anemic gave a mean value of 52.0% ± 9.26, and the untreated control group gave a mean value of 43.5% ± 10.04. The differences in the mean between concentrated anemic and untreated controls have limited significance at a 5-10% level (t – 1.909).

Discussion. The above data demonstrate as judged by Fe^{59} uptake into RBC, the erythropoietic stimulating property of a de-

TABLE IV. Normal Male Swiss Mice Given Deproteinized Plasma Extract (Lot #1) Subcutaneously from Anemic and Normal Rabbits for 3 Consecutive Days.

% uptake of Fe^{59}* in RBC, 48 hr after last inj. of plasma.

	Wt, g		% Ht.	% Fe uptake
Conc. anemic rabbit deproteinized plasma extract (13 mice)	29		46	50.6
	28.5		45	43.7
	28		46	45.3
	27.5		44	43.6
	22		46	42.7
	27		47	36.6
	23		46	38.6
	26		45	45.1
	28		44	32.2
	27		45	46.1
	19		48	30.1
	28		43	45
	23.5		—	23
		Avg	45.4	38.3
		S.D.	± 1.38	± 8.01
Conc. normal rabbit deproteinized plasma extract (12 mice)	32		41	31.4
	29.5		48	36.7
	27.5		43	34.6
	21		45	16.9
	25		45	27.9
	27		43	41.6
	28		45	22.8
	30		46	25.1
	30		42	42.4
	32		41	30.5
	29		46	19.6
	24		44	40.5
		Avg	43.7	30.8
		S.D.	± 1.77	± 8.6

* 1 μc Fe^{59} in ½ cc saline inj. intrav. 24 hr after last plasma inj.

t = 2.259. P = Statistically significant at a 2-5% level.

TABLE V. Normal Male Swiss Mice Given Deproteinized Plasma Extract (Lot #2) Subcutaneously from Anemic Normal Rabbits for 3 Consecutive Days.

% uptake of Fe^{59}* in RBC, 48 hr after last inj. of plasma.

	Wt, g		% Ht.	% Fe uptake
Conc. anemic rabbit deproteinized plasma extract (9 mice)	30		45	54.6
	33.5		42	58.5
	28.5		33	60.6
	25		43	55.3
	22.5		41	43.7
	22.5		42	47.4
	25.5		40	56.2
	25.5		45	32.3
	26		41	59.5
		Avg	41.3	52
		S.D.	± 3.57	± 9.26
Untreated controls (10 mice)	23.5		44	32.2
	22		39	26.1
	31		38	49.7
	24		43	45.6
	30.5		35	48.5
	31		40	60.6
	36.5		43	48.6
	34		38	36.8
	31		39	38.5
	27		40	48
		Avg	39.9	43.5
		S.D.	± 2.77	± 10.04

* 1 μc Fe^{59} in ½ cc saline inj. intrav. 24 hr after last plasma inj.

t = 1.909. P = Statistically significant at a 5-10% level.

proteinized plasma extract (Lot 1) from anemic rabbits. However, plasma extracts from anemic rabbits previously tested in normal and hypophysectomized rats(3) have been found to be much more effective with a smaller dose on a per gram body weight basis inasmuch as a dose 6 times greater to the mouse gave an erythropoietic response of lesser magnitude. It is interesting to point out here that in assaying growth hormone preparations in our laboratory we also repeatedly observe the greater sensitivity of the rat to growth hormone than the mouse. Nevertheless, it seems probable that the erythropoietic principle in anemic rabbit blood is effective generally in experimental animals. Keighley and Borsook(7) have shown the production of increased circulating hemoglobin in Swiss mice with extract from the plasma of rabbits made anemic by phenylhydrazine. Their study using hemoglobin determinations, although on a total of 12 animals, confirms our results using the Fe^{59} method. However, their erythropoietic effect from anemic rabbit plasma was obtained after a longer injection period of 7-20 days.

Fe^{59} studies with normal Swiss mice under biological conditions such as induced plethora which are known to affect the bone marrow's output of red cells in other experimental animals and humans(8,9) lends support to the concept that Fe^{59} uptake in the mouse is a valid representation of RBC production. Much the same responses with the Fe^{59} uptake method are seen in the mouse as are seen in other animals, and human studies(8-11) using the same method. Perhaps the Fe^{59} method might provide a method for studying erythropoietic dynamics and the effectiveness of chemotherapeutic agents on various blood disorders of the mouse. The mouse is an animal widely used in biological research and is readily available and economical to work with. Moreover, strain specificity control is available in the mouse to a much greater extent than rats and other available species of animals.

Summary. The above data demonstrate, as judged by Fe^{59} uptake into RBC, the erythropoietic stimulating property of a deproteinized plasma extract (Lot 1) from anemic rabbits in male Swiss mice. Fe^{59} studies with normal male Swiss mice under the experimental condition of induced plethora lends support to the concept that Fe^{59} uptake in the mouse is a valid representation of RBC production.

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Lung Area from Surface Tension Effects. (23155)

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A method for estimating the internal surface area of the lungs was recently presented by Radford which depends upon the thermodynamic properties of the surface(1). The free energy change in the lungs can be calculated from the static pressure-volume relationships during deflation. This change in free energy results from dissipation of surface free energy and elastic free energy as the volume decreases. When the lung is filled with and immersed in saline, the surface free energy is minimized because interfacial tension is small compared to surface tension. Therefore, the difference in the free energy between deflation in air and deflation in saline represents surface free energy. The elastic energy is assumed to be unchanged on passing from air to saline. Since the change in surface free energy is a product of surface tension and change in surface area, when 2 of these quantities are known the third can be computed. Unfortunately, 2 of these quantities are unknown, namely the surface tension and the change in area.

In his computations Radford assumed that the surface tension would be approximately that of serum, 50 dynes per cm. This assumes that the fluid on the lung surface is a pure transudate of the blood fluids. The surface area computed on this basis at functional residual capacity (FRC) was 5-10 m^2 for man. The discrepancy between this estimate and the histologic estimates of Willson(2) and Gertz(3) was attributed by Radford to distortion in fixation and errors in assuming a total lung volume. These factors when applied to the histologic estimates would produce values in the range of 7-20 m^2. Extracts of lung tissue were studied by von Neergaard (4) who obtained surface tensions of 35 to 41 dynes per cm. On the other hand, Pattle, on the basis of stability of bubbles expressed into air saturated saline from cut lung surfaces, concluded that lung surface tension approached zero(5). His view is probably somewhat extreme, since insoluble surface films hinder the transfer of materials across surfaces. Pattle demonstrated such a surface film, probably mucoprotein in nature, by staining of the stable bubbles. He produced similar stable bubbles from nasal and gastric mucus but not from blood, amniotic fluid or tracheal mucus. Because the surface tension decreases during compression of a protein film, both von Neergaard and Pattle could be correct; von Neergaard measuring the expanded film, and Pattle the maximally compressed film. This report presents evidence that such is indeed the case.

Results. The surface tension of nasal mucus films was 40-50 dynes per cm by the capillary tube method. A crude indication of the effects of compression was obtained by measuring the static pressure and volume in bubbles blown from nasal mucus, allowed to stand 15-30 minutes, and deflated by stages. Surface area was computed from volume and surface tension from pressure and volume. A marked decrease in surface tension was observed on deflation, reaching a limiting value of about 17 dynes per cm (Fig. 1). The minimum coefficient of compressibility or ratio of change in log of area to change in surface tension was 0.016 cm/dyne. Similar studies on human pulmonary edema fluid indicated a limiting value of 5-10 dynes/cm and when expanded 40-50 dynes/cm. This material, however, was not fresh when tested.

An *in vivo* deflation of the lungs of rats, cats and dogs provided pressure-volume relationships for calculation of lung surface tension. The tracheotomized animal whose pleural cavities were widely exposed was placed in a plethysmograph and respired with oxygen. After denitrogenation, the airway was clamped, and airway pressure and plethysmograph volume were recorded until apparently all gas had been removed from the lungs. Subsequently saline inflation and deflation were carried out on the degassed lung.

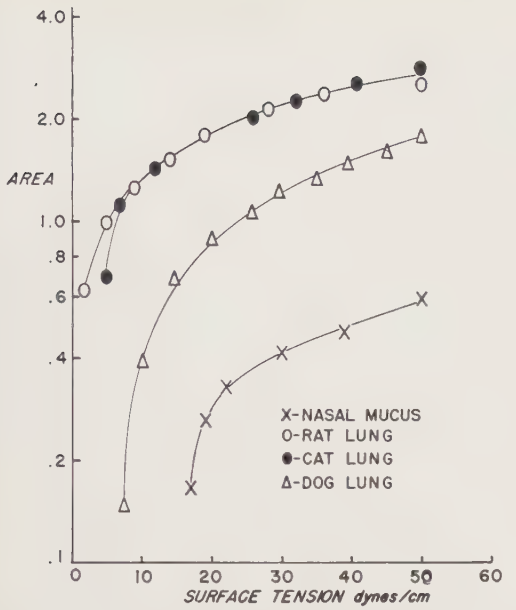

FIG. 1. Alteration of surface tension with change in area. Ordinate axis units: nasal mucus—cm^2; rat—.1 m^2; cat—1 m^2; dog—10 m^2.

The pressure-volume curves obtained by this procedure were similar to those obtained by von Neergaard and by Radford. The saline deflation curves were extrapolated to zero volume since complete emptying was not effected. The differential pressure at the end of air deflation was –1.5 to –5 cm of water. Little or no change in rate of oxygen uptake was observed during the course of deflation. However, cardiac output was not measured, so changes in oxygen transfer coefficient could not be noted.

A lung volume was selected which was near full inflation but still in the linear region of the pressure-volume curve. The surface tension after inflation to this volume was assigned a value of 50 dynes/cm for purposes of computation. A small deflation from this volume was assumed to decrease all spatial coordinates of the lung units proportionately and, hence, that surface area was proportional to volume to the two-thirds power. From the free energy change appearing as the area between the air and saline curves and the assigned surface tension, the change in surface area was calculated. The total surface area was computed using the assumed proportionality between surface area and volume to the two-thirds power. From this area, areas at other volumes were calculated from this proportionality.

The surface tensions relative to the assumed 50 dynes/cm value were calculated at other volumes from the free energy change and the change in calculated area. The relative surface tension in rat, cat, and dog lungs decreased rapidly with deflation (Fig. 1). The limiting value for relative surface tension was 5 to 10 dynes per cm. The minimum coefficients of compressibility ranged from 0.012 to 0.020 cm/dyne as compared to 0.016 cm/dyne for nasal mucus.

While absolute values cannot be assigned to the surface tension at a particular lung volume, the surface tension in the expanded lung probably is not smaller than the 35 to 41 dynes per cm found by von Neergaard. The lower limit is in doubt because of extrapolation of the saline pressure-volume curves but probably is not zero. Intermediate values should obtain at functional residual capacity and a small oscillation in surface tension probably occurs with quiet breathing. Because rate of oxygen uptake remained essentially constant during deflation, resistance of the lung surface film to oxygen transfer is probably not limiting except when severely compressed.

The lung surface areas at functional residual capacity calculated for rats, cats and dogs were linearly related to body weight. Extrapolation to a body weight of 70 kg indicated a surface area of 70 square meters for man. A simple computation of mean diameter of a lung unit was made by assuming these units to be hemispherical. The mean diameter is 6 times the volume to area ratio. These mean diameters (Table I) were approximately twice those found by Macklin and Hartroft(6). A further calculation using the diameters of Macklin and Hartroft gave the number of "lung units" required to yield the calculated surface area.

The aggregate picture of the human lung as developed in these computations is in good accord with previous histologic estimates. A surface area of 70 m^2 at a functional residual

TABLE I. Lung Surface Area and Alveolar Parameters in Several Species.

	Wt (kg)	FRC (cc)	Area (m^2)	Dia. alv. (calc.), μ	Dia. alv. (obs.*), μ	No. of alv. (millions)
Rat	.2	5	.18	167	60	8.0
Cat	2.	100	2.075	290	130	19.5
Dog	10.	400	10.3	230	75	290.
Man	70.	2250	70.	190	150†	500.

* From Macklin and Hartroft(6). † Recalculated for 2250 cc lung vol.

capacity of 2250 cc is associated with an alveolar volume of 1750 cc comprising about 80% of the total volume. The number of alveoli is 500 million and—from Macklin and Hartroft—their mean diameter is 150 μ. The surface film has a surface tension of 20-25 dynes/cm. The surface tension rises to 40-50 dynes/cm on maximum inspiration and falls to 15-20 dynes/cm on expiration to residual volume.

Summary. The surface tension of pulmonary edema fluid was 5-10 dynes/cm as measured in aged, compressed bubbles; it was 40-50 dynes/cm as measured by capillary tube. Nasal mucus on aging and compression exhibits a fall of surface tension from 45-50 dynes/cm to 17 dynes/cm with a minimum compressibility coefficient of 0.016 cm per dyne. Calculations of relative surface tension in lungs of rats, cats and dogs assuming 50 dynes/cm for the upper limit showed a fall to 5-10 dynes/cm during deflation with minimum compressibility coefficient of 0.012 to 0.020 cm/dyne. The lung surface areas calculated for these species were proportional to body weight and extrapolated to 70 m^2 for a 70 kg man.

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Surface Tension of Lung Extracts. (23156)

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Von Neergaard described the important role played by surface forces in the recoil of the lungs and made measurements of surface tension in lung extracts(1). Recently, Radford and his coworkers repeated and extended von Neergaard's experiments, again calling attention to the importance of surface tension in the static pressure-volume characteristics of the lungs(2) and pointing out the need for proper determination of the surface tension of the lung lining. In the interim Radford used the so-called static tension of serum (50 dynes/cm) in interpreting his results, assuming the surface to be thermodynamically reversible. From microscopic study of air bubbles squeezed from lung slices Pattle(3) concluded that the pulmonary alveoli are lined with mucoprotein (suggested previously on the basis of histochemical evidence by Macklin(4)) and that this material reduced the surface tension to less than 1 dyne per cm. In the last year Brown(8) repeated Radford's experiments and by assuming the lung to be composed of many identical hemispherical units computed surface tension from the pressure-volume data. The tension-area relationship so derived is similar to that of bubbles of nasal mucus, but depends upon an assumed

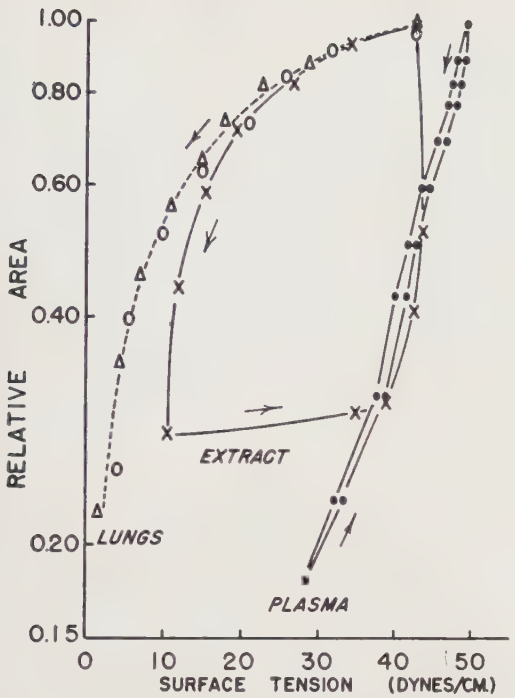

FIG. 1. Variation of surface tension with surface area. Upper curve (lungs) calculated on the basis of relative area from Brown's data. Large loop constructed from measurements on lung extract in Wilhelmy balance. Narrow loop constructed from measurements on blood plasma in Wilhelmy balance.

area-volume function ($A = KV^{2/3}$) which is not acceptable if the lung units deviate significantly from the mean radius. In addition Brown's calculations did not correct for the tendency of the units to close off above zero volume as the transpulmonary pressure is reduced. To obtain more direct evidence bearing on the above points, we have studied the tension-area behavior of lung-derived surfaces, using modifications of the Langmuir-Adam film balance and the Wilhelmy balance (5). Surfaces of the following fluids were examined: a. normal saline (0.85% NaCl) after it had been used to inflate degassed lungs via the trachea; b. mince of whole lungs in normal saline, filtered through loosely-packed cotton; c. normal saline, to which slices of lung parenchyme had been touched. These were prepared from rat, cat, and dog lungs.

Results. The results were similar in all cases. A typical tension-area plot is shown in Fig. 1. This figure demonstrates that the tension of the lung-derived surface varied from 46 to 10 dynes/cm as its area was changed, and further that the surface exhibited extreme hysteresis, although 80 minutes was used for the compression-expansion cycle. The same pattern was obtained when the cycle was repeated. Thus, the mechanical behavior of the surface was far from reversible, within the duration of most pressure-volume measurements on lungs.

The coefficient of compressibility $\left(\frac{1}{A}\frac{dA}{d\gamma}\right)$ of these surfaces ranges from 0.010 to 0.025 cm/dyne at the higher tensions, agreeing well with Brown's values. This characteristic of the surface has a stabilizing influence and might be called an "anti-atelectasis factor." At lower tension the surface compressibility rises and closure of lung units becomes probable. It is in this range that Brown's data depart from the extract data, signalling trapping of gas within the lungs(6).

We have examined Pattle's conclusion that the surface tension of his "alveolar bubbles" (about 40 μ diameter) and hence of the pulmonary alveoli was less than 1 dyne/cm. While repeating his experiments we found that lung bubbles which were "stable" in static air-saturated saline, dissolved slowly when the saline continuously perfused the microscope chamber. Determining increments of hydrostatic pressure necessary to double the instantaneous rates of solution permitted calculation of the surface tension of the bubbles. Although the method was crude, it gave values from 10 to 15 dynes/cm. Bubbles of the same diameter prepared in an air-saturated soap solution having a surface tension of 27 dynes/cm dissolved rapidly. The transfer coefficients were 9.3×10^{-5} and 1.7×10^{-3} cc/cm^2 -atm.-sec., respectively. Taking the difference of the reciprocals we estimated the specific diffusion resistance of the lung bubble surfaces at 1.0×10^{4} sec/cm; this is much higher than the specific resistance of compressed films of 18- to 20-carbon fatty acids to water diffusion found by Archer and LaMer(7). Thus the slow solution of the lung bubbles appears due mainly to the diffusion characteristics of the surface. Cal-

culation shows that if this surface existed in the lungs, it would more than account for the diffusion resistance at rest. Measurement of the relationship between surface pressure and diffusion resistance of lung-derived films is indicated.

Summary. Saline-extractable surface-active material has been found in the lungs of rat, cat, and dog. This material, probably mucoprotein, imparts large hysteresis and characteristic elasticity to the fluid surface. Its effect on lung mechanics has been studied. Its possible influence on diffusion across the alveolar barrier remains to be elucidated.

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^{14}C Studies on Ketogenicity of Metabolites in Lactating Dairy Cows.*† (23157)

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Recent studies upon the intermediary precursors of B-hydroxybutyric acid, acetoacetic acid and acetone in the intact dairy cow have been mainly concerned with ability of the administered compound to elevate the serum and urinary ketones(1-6). Extensive investigations have been made by Kleiber *et al.* (7-10) concerning transfer of carbon-14 of intravenously injected metabolites into milk casein, lactose and fat. In these trials ^{14}C-labeled carbonate, the lower fatty acids from formate to caproate, norleucine, and glucose were injected into normal lactating dairy cows and efficiency of transfer(11) to milk products calculated. Since up to 85% of milk lactose may originate from the plasma glucose(12), incorporation of relatively large numbers of carbon-14 atoms from a metabolite into lactose should indicate glucogenicity. Entrance of carbon-14 atoms into milk fat from an injected metabolite is a measure of its role in lipogenesis and not necessarily its ketogenicity. The present study was undertaken to measure relative transfer of carbon-14 atoms of injected compounds into the urinary ketone bodies.

Materials and methods. Mature lactating dairy cows were injected intravenously with glycine-1-^{14}C, butyrate-2-^{14}C, norvaline-3-^{14}C, carbonate-^{14}C, glucose-1-^{14}C, and propionate-2-^{14}C by methods previously reported by Kleiber(11). The urine was voided at the time of administering the isotope and then collected for the first hour following the injection for preparation of the ketone bodies. Two hundred fifty ml of urine was first treated with 58 ml of a solution containing 1.5% $K_2Cr_2O_7$ in 15.6N H_2SO_4 to convert the B-hydroxybutyric and acetoacetic acid to acetone(13). Another 50 ml of 5% $K_2Cr_2O_7$ was added after boiling had commenced to insure complete oxidation(14). The carboxyl carbon of B-hydroxybutyric and acetoacetic acid was not recovered. The mixture was next doubly distilled first into cold distilled water and secondly into 150 ml of Deninges' solution(15), adding 1 g Na_2O_2 per 100 ml of distillate prior to the second distillation to insure complete oxidation of

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TABLE I. Summary of Data from Trials.

Metabolites inj. intrav.	μc inj./kg body wt	g-atoms of acetone carbon* per 100 ml urine	μc/g atom acetone carbon	Stand. specific activity† of acetone carbon	Relative ketogenicity‡
Propionate-2-^{14}C	8.83	1.62×10^{-4}	3.77	6.92×10^{-5}	1.00
Carbonate-^{14}C	9.01	7.36×10^{-4}	.87	7.12×10^{-5}	1.03
Glucose-1-^{14}C	1.78	6.18×10^{-4}	.92	3.20×10^{-4}	1.80
Glycine-1-^{14}C	6.94	1.77×10^{-3}	.51	1.30×10^{-4}	1.88
Butyrate-2-^{14}C	9.02	3.53×10^{-4}	126.0	4.93×10^{-3}	71.3
Norvaline-3-^{14}C	4.12	1.88×10^{-4}	224.2	1.02×10^{-2}	148.0

* Acetone carbon includes only carbon originating from the 2, 3, 4 carbon atoms of urinary B-hydroxybutyric and acetoacetic acid in addition to urinary acetone carbon.

† μc/100 ml urine/μc inj./kilo body wt.

‡ Relative entrance of carbon-14 atoms from a given precursor into urinary acetone carbon* as compared with propionate-2-^{14}C.

acetaldehyde and other related compounds (14). To insure absence of any extraneous carbon from compounds other than acetone, acetone-2-^{14}C was added to urine of known acetone concentration containing no radioactivity. No measurable extraneous carbon was present in the second acetone distillate. The acetone-mercury-sulphate-chromate precipitate was prepared according to Van Slyke (15) for quantitative urinary acetone measurements and subsequently combusted to CO_2 according to the method of Van Slyke and Folch(16). In the glycine-1-^{14}C trial, iodoform was also prepared for combustion from the acetone-mercury-sulphate-chromate salt as described by Weinhouse *et al.*(17); in this method only the 1 and 3 carbon atoms of acetone are converted to iodoform. $BaCO_3$ planchets were prepared from the combusted samples as described by Kleiber and Edick (18) and counted for radioactivity at infinite thickness with a Tracerlab windowless Geiger flow gas counter and autoscaler.

Results. Standard specific activities were calculated to compare the ketogenicity of the injected metabolites. These results are presented in Table I. Both norvaline-3-^{14}C and butyrate-2-^{14}C are highly ketogenic as contrasted to propionate-2-^{14}C, glucose-1-^{14}C, glycine-1-^{14}C and carbonate-^{14}C. The relative ketogenicity of one carbon-14 atom in a metabolite need not reflect ketogenicity of other carbon atoms in the same molecule. In the glycine-1-^{14}C trial, all of the measurable radioactivity was confined to carbon-2 of the acetone since the iodoform containing the methyl carbon atoms exhibited only background levels of activity upon combustion.

Discussion. Kleiber *et al.*(7-10) have shown that propionate and glucose contribute greatly to lactose synthesis. Since the majority of the lactose originates from plasma glucose(12), entrance of carbon atoms into lactose from these precursors may be used as an index for their relative contribution to glucose formation, their "glucogenicity." Lardy *et al.*(19), using soluble enzymes from beef liver mitochondria, have recently explained the glucogenicity of propionate and bicarbonate by showing the synthesis of succinate from these two metabolites. The glucogenicity of succinate as a TCA intermediate is well known. The metabolism of glycine to the intermediate serine (20,21) and subsequently to pyruvate also leads to glucose formation. Compounds metabolized mainly to glucose generally contribute little to the formation of ketone bodies. Table I shows the relatively low ketogenicity of propionate, carbonate, glucose, and glycine but shows a relatively high ketogenicity for butyrate. Butyrate, however, also exhibits a glucogenic behavior. Blixenkrone-Möller(22) observed increases of glycogen in feline livers perfused with butyrate. This was not direct evidence for the incorporation of butyrate carbon atoms into glycogen since no tracers were used, but was conclusively a net synthesis of glycogen. After injection of butyrate-2-^{14}C into lactating cows, Kleiber *et al.*§ observed a maximum specific activity

§ Reported at International Physiol. Congress, 1956, 503.

in the plasma glucose 140 times as high as that of blood acetate and higher ^{14}C activity in the lactose than in the butterfat. Much of the acetyl-CoA produced by beta-oxidation of the butyrate-2-^{14}C could well be incorporated into glucose within the hepatic cell via the TCA cycle since glucose is withdrawn rapidly for lactose synthesis(12). After injection of norvaline-3-^{14}C, maximum specific activity of the plasma glucose was 98 times as high as that of the blood acetate.¶ Norvaline, as indicated in Table I, also has a relatively high ketogenicity. Both norvaline and butyrate exhibit glucogenic behavior in addition to showing a relatively high ketogenicity as compared with the other metabolites investigated.

Summary. The relative transfer of carbon-14 from propionate-2-^{14}C, carbonate-^{14}C, glucose-1-^{14}C, glycine-1-^{14}C, butyrate-2-^{14}C, and norvaline-3-^{14}C into the 2, 3, 4 carbon atoms of urinary B-hydroxybutyric and acetoacetic acid, and acetone has been determined. Carbon-14 atoms of norvaline-3-^{14}C and butyrate-2-^{14}C were more ketogenic than those present in the other compounds investigated.

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Effects of Low Doses of X-Rays on Embryonic Development in the Mouse. (23158)

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Irradiation of mice in various stages of pregnancy has shown that the effect observed in the young subsequently born is closely correlated with the embryonic stage at which they were irradiated. Irradiation during the first 5 days, *i.e.,* prior to the embryo's implantation in the uterus, leads to all-or-none effects: there is a high incidence of death shortly after irradiation but those embryos that survive are normal. On the other hand, irradiation during the next 8 days, the period of major organogenesis, has little effect on

* Operated by Union Carbide Nuclear Co. for U. S. Atomic Energy Com.

survival to birth of the embryos, but almost all the animals born are abnormal, the exact type of malformation depending closely on the exact stage reached at the time of irradiation(1-6). All of our detailed reports to date have concerned experiments in the range from 100-400 r, and particularly doses of 200 and 300 r. From comparisons within that range it was quite evident that there must be different types of dose-effect curves for the production of different abnormalities at the respective sensitive stages. For the purpose of getting more extensive information on that point, lower dose ranges were chosen for the following reasons: (a) the total number of abnormalities in a given animal is smaller and the chance of abnormalities interfering with one another in expression therefore reduced; (b) while no threshold in production of certain abnormalities could be detected in the higher dose range, lower doses were needed to show whether or not the dose-incidence relation in such cases was truly continuous; and (c) from the point of view of hazards of radiation to human beings, it seemed desirable to have direct experimental evidence in a dose range occasionally encountered in fluoroscopy (7,8).

Because evidence on the third point is of practical importance, a preliminary report of the data is presented here, although analysis of the large amount of material collected(9) is not yet complete.

Material and methods. The developmental stages at which radiation was applied in the present experiment were 2 that, on the basis of our earlier results(2), were expected to yield not only malformations but also several quantitative changes of a type suitable for certain kinds of analysis. These quantitative effects include homeotic changes in the axial formula, and changes in the number of costo-sternal junctions and number of sternebrae. Day 8½ postconception has been shown to be a stage highly sensitive to the induction of these changes by radiation(2). Day 7½ is somewhat less sensitive to the same quantitative changes; it is, on the other hand, highly sensitive to the induction of various malformations of the axial skeleton.

The axial formula is variable within each of a number of inbred strains of mice studied (10). The mean value is characteristic of each strain, indicating that genetic constitution determines location of the strain on a scale of developmental potencies, while environmental factors cause individuals to be distributed about this mean. Although the final expression of the character is discontinuous (*e.g.*, either 25 or 26 presacral vertebrae) due to developmental thresholds, the continuous distribution in underlying processes can be mathematically derived(11). The strain chosen for the experiment here reported was the *BALB/c*, whose position with regard to the thresholds is such that even small induced shifts should be readily detectable; *i.e.*, the strain is naturally variable in the position of the thoraco-lumbar border, lumbo-sacral border, number of costo-sternal junctions, and number of sternebrae.

Females of the *BALB/c* strain were irradiated with 250 kvp X rays either 7½ or 8½ days after observation of a vaginal plug (following mating with *BALB/c* males). Doses used were 25, 50, and 100 r. Control females were taken through all the motions of the irradiation procedure. Litters were allowed to come to term. Newborns were killed, observed for various external and visceral characters, then processed for skeletal observation. Details of the technic have already been described(1,2). Skeletal observation was for vertebral column and thorax only. To date, 253 skeletons have been studied.

Results. Although both malformations and quantitative characters were recorded for each animal studied, this preliminary communication will present the former only for animals irradiated day 7½ postconception (Table I), the latter only for animals irradiated day 8½ (Fig. 1).

1. Malformations. Two broad conclusions may be drawn from the results shown in Table I. First, it is apparent that the incidences of all but one of the abnormalities (simple split of thoracic centra) increase with dose, and that dose curves for individual abnormalities are of a variety of shapes (compare, *e.g.*, "jumbling" of cervical centra with

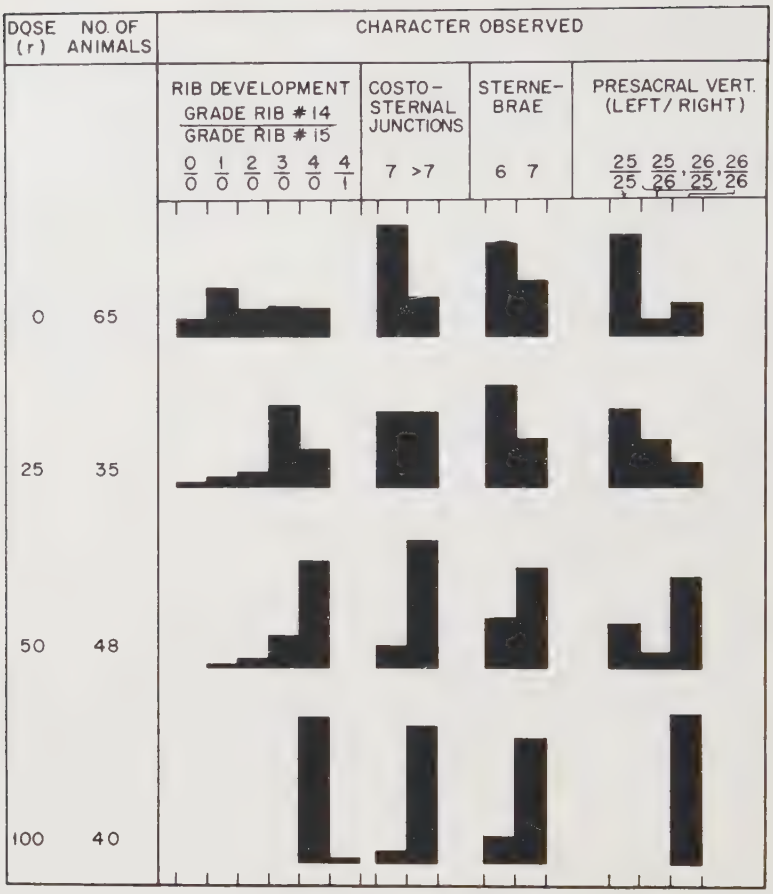

FIG. 1. Effects of irradiation on day 8½ postconception on various quantitative skeletal characters observed at birth in *BALB/c* mice. Histograms indicate percentages in the various categories.

fusion of ribs). It should, however, be pointed out that there are complications in making dosage comparisons. For one thing, a complete dosage comparison would have to take degree of abnormality into consideration. This has not been attempted here, Table I revealing only the incidence. Furthermore, it is quite likely that several of the abnormalities are correlated(2). Some of the abnormalities classified separately may not really be separate at all but merely represent different degrees of expression of the same basic effect.

A second conclusion that may be drawn from the results shown in Table I is that the lowest dose used in the present experiment, 25 r, gives effects that are detectable even with the relatively small sample sizes of this experiment. In the 25 r-group, the incidence of all 5 of the abnormalities normally found in *BALB/c* was increased and, in addition, 6 abnormalities were observed that were not present in controls. Although none of the *individual* comparisons that can be made is statistically significant by itself, comparison of the groups as a whole yields highly significant results. This is true regardless of which one of two opposite and extreme assumptions are made. Thus, if one considers all malformations completely uncorrelated with one another, *i.e.*, each embryo as the site of 17 possible independent malformations, the control group has 27 malformations in a total of 657 possible ones, while the 25 r-irradiated group has 57 in 507 possible ones ($\chi^2 = 20.69$, $P<0.001$). If one goes to the other

TABLE I. Percentage Incidence of Abnormalities of Axial Skeleton following Irradiation of Strain *BALB/c* Mouse Embryos 7½ Days Postconception.

Abnormalities†	Dose 0 r	25 r	50 r	100 r
	No. animals obs.* 39	30	29	6
Cervical				
atlas, arcus anterior	0	13.3	13.8	100
" , lateral masses	0	13.3	24.1	100
axis, dyssymphysis	23.1	43.3	48.3	100
centra,‡ reduced	5.4	17.2	19.2	75
" ,‡ absent	0	13.8	26.9	100
" , "jumbled"	0	3.4	23.1	66.7
arches, "	0	0	3.4	66.7
" , miscellaneous	0	0	3.4	16.7
Thoracic				
centra,‡ simple split	25.6	33.3	38.0	33.3
" ,‡ uneven split or split with reduction	5.1	20.0	41.4	100
" , "jumbled"	0	0	0	100
arches, "	0	0	0	83.3
" , other	0	0	0	16.7
Lumbar				
centra,‡ split	10.3	26.7	38.0	80
Thorax				
ribs,§ fused	0	0	0	100
costal cartilages,§ fused	0	3.3	10.3	100
sternum, "jumbled"	0	3.3	17.2	100

* A few percentages in the Table are based on somewhat lower numbers, since a few skeletons were damaged in certain regions or were otherwise unobservable.

† Descriptions of most of these abnormalities may be found in an earlier publication(2).

‡ One or more.

§ Two or more.

extreme and makes the most conservative assumption, namely, that all malformations are completely correlated (which is obviously not the case), the comparison between the groups is made on the basis of animals with or without malformations, namely, 21 versus 18 in controls, 26 versus 4 in 25 r-irradiated animals ($\chi^2 = 6.97$, $P<0.01$). (On the same assumption, but omitting from the abnormal classification those animals that have no abnormalities other than simple split of thoracic centra—the one character apparently not affected by increasing the dose—the significance of the difference between the control and 25 r-groups is increased: $\chi^2 = 9.75$, $P = 0.003$). The true case lies somewhere between the extremes set up here; *i.e.*, there is probably some correlation between abnormalities(2), but this correlation is far from complete. Since the difference between the control and 25 r-irradiated groups is significant even with the most conservative assumption, it is clear that 25 r gives effects that are detectable in samples of the size used here.

2. Quantitative changes. All of the 4 characters shown in Fig. 1 are clearly affected by irradiation on day-8½ postconception. In all cases, the effect increases with dose and in 3 of the 4 it is already apparent after only 25 r. The difference between the 25 r-group and the controls is highly significant, both with respect to rib development and to the number of costo-sternal junctions ($P<0.001$ and $P = 0.0018$, respectively). The effect of 25 r on number of presacral vertebrae is on the borderline of significance ($P = 0.037$) when the data are divided into 3 categories (25/25; asymmetrical, *i.e.*, 25/26 and 26/25; 26/26).

Discussion. Low dose irradiation of mouse embryos, at stages known from our earlier work to be sensitive to induction of certain abnormalities in vertebral column and thorax, has shown (a) that there are different types of dose-effect curves for different abnormalities, and (b) that a dose as low as 25 r gives effects that are detectable even in relatively small samples.

Analysis of dose-effect curves, when completed, will contribute to our knowledge of mechanisms of radiation effect on developing organisms. The sensitivity of the system, as demonstrated in this experiment, gives it a valuable place among the few biological systems that can be explored in the low dose range.

The results must also be examined from the point of view of human hazards. Individual skeletal abnormalities that may be called drastic are found only with low incidence after 25 r. However, the average number of minor skeletal defects per animal is markedly increased. Since only one organ system was examined here, it seems likely that the overall effect of 25 r on all organ systems would add up to a certain load on the individual. The present results, therefore, reinforce our earlier recommendation(5,6) that, whenever possible

pelvic irradiation of women of childbearing age should be restricted to the 2 weeks following menstruation when the chance of an unsuspected pregnancy is small. This recommendation is based on the fact that the stages found most vulnerable in the mouse correspond developmentally to weeks 2-7 in human embryonic development, *i.e.*, to stages so early that pregnancy may still be unsuspected. The earlier work was primarily in the 200-300 r range(1-4) and recommendations for the human case were made mainly by downward extrapolation, supported, however, by some data from 100, 50, and 25 r irradiations(9). The present data, forming an extension to the former meager information at 25 r, are of especial interest to the human hazard problem since they deal with a dose level that is, on occasions, actually encountered in medical practice.

Summary. Mouse embryos of the *BALB/c* strain were irradiated 7½ or 8½ days postfertilization with 25, 50, or 100 r of X rays (doses lower than any heretofore investigated in detail), allowed to come to term, and then observed for changes in vertebral column and thorax. It was found (a) that there are different types of dose-effect curves for different abnormalities, and (b) that a dose as low as 25 r gives effects that are detectable even in relatively small samples.

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Elemental and Amino Acid Composition of Purified Plague Toxin. (23159)

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(Introduced by Geoffrey Edsall)

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During the past several years, we have been concerned with studies on the production, purification, characterization, and mechanism of action of the murine toxin of *Pasteurella pestis*(1,2). The isolation of the toxin in purified form(1) has led to an interest in its chemical composition. A detailed analysis of the purified material is presented in this report.

Materials and methods. Strain "Tjiwidej" (TJW) of *Pasteurella pestis* was employed for toxin production. The organisms were grown either in trypticase soy broth and the toxin extracted from the cells or in a casein hydrolysate-mineral-glucose medium(3) and the toxin salted out from solution after autolysis of the bacteria. For all analytical determinations, the toxin was purified either by the method described by Ajl, *et al.*,(1) or by the large scale ionophoresis procedure of Karler. Criteria of purity included homogeneity of the material in the ultracentrifuge and electrophoresis cell(1). By the gel precipitation technic there were a major component and a small minor component in the preparation used for most of this work; a sub-

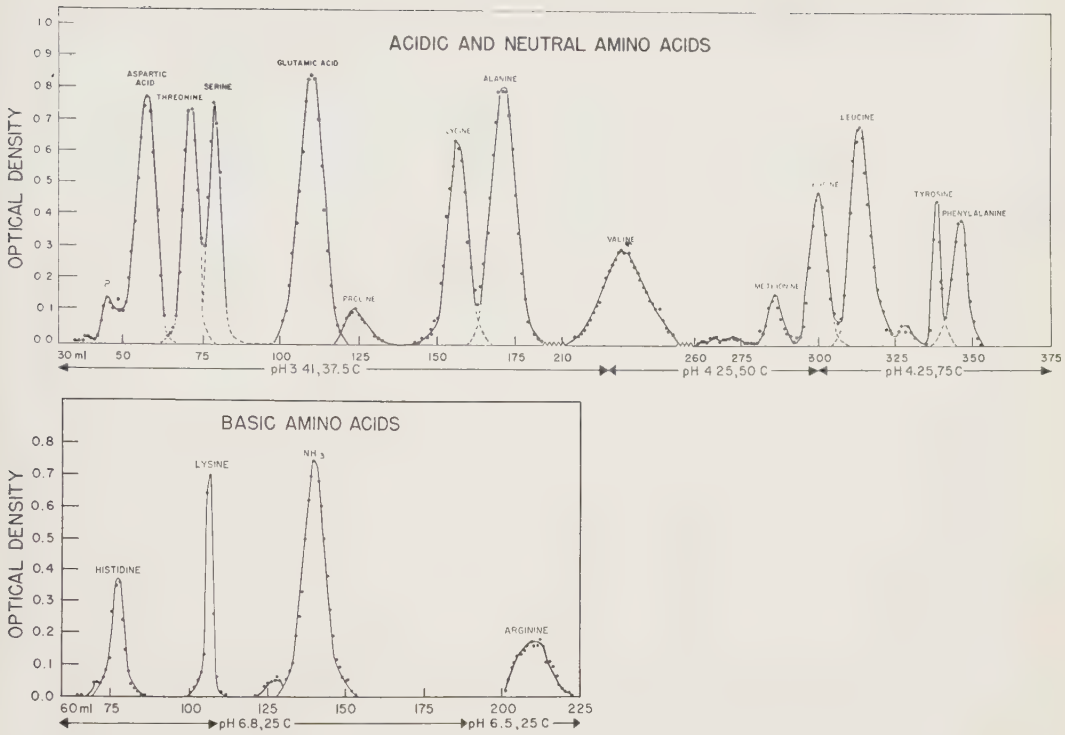

FIG. 1. Separation of amino acids of purified plague toxin by means of chromatography on ion-exchange resins.

sequent toxin preparation with only one band by the Oudin technic was analyzed for amino acid content and was found to differ in no measurable respect from the toxin lots used for the basic work reported here. Amino acid analysis was performed on weighed samples of toxin which were hydrolyzed in 6N HCl in sealed tubes at 110°C for 10 hours. The amino acids were separated on Dowex-50 ion-exchange resin columns according to the method of Moore and Stein(4) and quantitatively estimated by the ninhydrin procedure of Yemm and Cocking(5) as modified by Rosen.* To avoid the destructive action of acid hydrolysis, tryptophane was determined on the intact protein by the colorimetric method of Spies and Chambers(6). Cysteine plus cystine (calculated as cysteine) was estimated by first oxidizing these acids in the intact toxin molecule to cysteic acid with performic acid(7). The treated toxin was then hydrolyzed in the conventional manner and the cysteic acid separated on the Dowex-50 column. In several instances, the concentration of methionine was determined on the hydrolyzed protein by the nitroprusside method of Horn *et al.*(8). Moisture was determined by heating known samples of toxin to a constant weight in a drying pistol. Ash was determined by pre-charring under infra-red and ashing at 550°C. Nitrogen was determined by micro Kjeldahl. All inorganic elements were identified spectrographically.

Results. A typical analytical run representing the relative concentrations of each amino acid of hydrolyzed toxin is shown graphically in Fig. 1. The total concentration of each amino acid is represented by the area under each curve and was calculated by the use of a planimeter. Appropriate factors, determined from identical analyses of standard amino acid solutions, were applied in order to convert the planimeter readings to actual concentrations.

A summary of the bulk of the data obtained in this study is shown in Table I. The

* To be published.

TABLE I. Amino Acid Composition of Purified Plague Toxin.

Amino acid	Wt of amino acid/100 mg protein: Lot No. 101	Lot No. 103	Avg of other lots	Calculated % amino acid N.	g moles of amino acid per 10^4 g of protein	Min molecular wt of amino acid residues	Residues of each amino acid per molecule*
Aspartic acid	10.5	10.4	10.9	1.34	94.6	1007	76
Threonine	7.0	7.0	7.2	1.00	71.2	693	54
Glutamic acid	11.8	12.2	12.1	1.30	93.6	1060	71
Serine	4.2	5.1	5.4	.90	62.0	484	47
Proline	3.2	3.8	3.5	.50	36.1	340	28
Glycine	4.2	4.0	4.1	1.00	71.8	359	55
Alanine	6.8	6.4	6.9	1.40	97.0	605	74
Valine	5.4	5.9	5.5	.78	55.5	482	43
Methionine	1.1	1.7	1.5	.16	11.4	131	9
Isoleucine	4.3	4.3	4.3	.53	38.0	377	29
Leucine	6.8	8.1	7.8	.96	68.9	685	52
Tyrosine	3.2	3.2	3.2	.50	19.6	280	15
Phenylalanine	5.0	5.2	5.3	.98	36.0	465	28
Histidine	3.5	3.0	3.2	1.16	23.3	280	17
Lysine	6.1	5.3	5.3	1.79	41.4	465	31
Arginine	5.8	4.6	5.0	.28	32.0	439	24
Tryptophane	3.8	2.7	3.3	.50	17.7	279	13
Cysteine + cystine	1.3	0.9	1.3	.18	12.6	114	10
NH_3	2.1	2.3	2.3	2.0			
Totals	96.1	96.1	98.1	17.26		8445	676

Total ash content = 1.9%. Total moisture = 11.6%.
* Based on molecular wt of 74,000(1).

method of presentation of the results follows that suggested by Brand and Edsall(9). Data are presented for 18 amino acids which together with the ammonia represent a recovery of over 98% of the hydrolyzed toxin. The values given have been corrected for moisture and ash content. The threonine and serine values have been increased by 10% as is the usual procedure to correct for their partial loss during acid hydrolysis. In calculating the residues of each amino acid per protein molecule, a molecular weight of 74,000 was assumed(1).

The elemental analysis of purified plague toxin† is shown in Table II. In addition to the relatively large amounts of sulfur(1), 19 elements have been positively identified. They are grouped and listed in order of decreasing concentration. Sodium, calcium and magnesium, therefore, can be considered of quantitative significance. Others such as lead, tin, silver, etc., are present in such small amounts that they could be considered of little or no importance as constituents of plague toxin.

It was reported(1) that upon hydrolysis, the toxin yields, in addition to the various amino acids, materials which absorb and fluoresce in the ultraviolet range. The problem of the nature of the fluorescent components was further investigated in conjunction with the amino acid analyses. A fluorescent compound was eluted from the Dowex-50 column along with the basic amino acids. Tryptophane, alone of the basic amino acids, is destroyed by acid hydrolysis and is not recoverable as such by the chromatographic procedure employed. Accordingly, a pure solution of tryptophane was hydrolyzed and chro-

TABLE II. Elemental Analysis of Purified Plague Toxin.

Elements		
Major (1-10%)	Minor (.1-1%)	Traces (.01-.1%)
Sodium	Boron	Silver
Calcium	Barium	Cobalt
Magnesium	Chromium	Manganese
Silicon	Copper	Titanium
Strontium	Nickel	Cobalt
Zinc	Phosphorus	
Aluminum	Lead	
Iron	Tin	

† Performed by the National Bureau of Standards, Washington, D.C.

matographed in the same manner as the toxin; this yielded a fluorescent fraction which emerged at the same position as the unknown fluorescent material. This suggests that the fluorescent product obtained from hydrolyzed plague toxin may be a degradation product of tryptophane.

Summary. A detailed elemental and amino acid analysis was performed on highly purified *Pasteurella pestis* murine toxin. Eighteen amino acids and a number of elements were identified. The high proportions of acidic amino acids found are in accordance with the previously observed isoelectric point of the toxin of 4.7(1). On a dry weight basis, 98% of the toxin molecule was accounted for by the organic analysis including ammonia and ash content.

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Electrophoretic Distribution of Serum Protein and Glycoprotein in the Tuberculous Rat, Rabbit and Guinea Pig.* (23160)

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Serum protein distribution changes in man and experimental animals during tuberculous infection have been the subject of several investigations(1-2). Likewise, alterations of serum glycoprotein concentration in tuberculosis have been of interest(3-5).

The present study attempts to compare serum protein and glycoprotein response of the natively resistant rat and the susceptible guinea pig and rabbit following infection with tubercle bacilli. In a previous study(6) we compared the response of these species to an attenuated (BCG) strain of tubercle bacilli.

Methods. Eight male albino rats, Sprague-Dawley (avg wt 350 g), 8 male albino guinea pigs (500 g) and 6 male albino rabbits (1200 g) were inoculated intraperitoneally with 0.1 mg (wet wt) of *Mycobacterium tuberculosis,* human strain H 37Rv. Six other rabbits were similarly injected with bovine strain (Ravenel) tubercle bacilli. All animals were bled before and 4 weeks following infection. The sera were stored at –20°C and control and experimental sera were analyzed in duplicate at the same time. Electrophoretic separation of serum protein and glycoprotein was done by a paper strip technic previously described (6), with the exception of using 8 Ma of current for electrophoresis instead of the former 10 Ma used. The electrophoretic strips for serum glycoprotein determination were stained by a method described by Roboz(7). Stained strips were scanned in the Spinco Analytrol with a green (Wratten XI) filter. Relative % distribution of glycoprotein was determined in this manner. Total (non-glucosamine) polysaccharide content of serum was measured by the method of Shetlar(8). Milligram % glycoprotein concentration of each protein fraction was determined from total polysaccharide and relative % distribution of glycoproteins

* The authors are indebted to Dr. M. M. Cummings and Dr. M. R. Shetlar for helpful suggestions in planning this study.

TABLE I. Electrophoretic Distribution of Serum Protein in Rabbits, Guinea Pigs and Rats following Inoculation with Virulent Tubercle Bacilli.

Group	Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	Total protein
	g %					
Normal rat	1.72	1.00	.89	1.71	1.18	6.53
H 37Rv	1.71	.97	1.06	1.34	1.37	6.45
"t" value	.1	.5	5.6 *	7.8 *	3.8 *	.5
Normal rabbit	3.86	.72	.36	.85	1.01	6.82
H 37Rv	3.11	.69	.47	.88	2.21	7.39
"t" value	5.7 *	.6	3.0 *	.3	7.0 *	6.2 *
Normal guinea pig	2.88	.29	1.89	.74	.45	5.99
H 37Rv	2.49	.51	1.73	.91	.88	6.55
"t" value	3.2 *	8.1 *	.8	4.6 *	7.5 *	5.6 *
Normal rabbit	3.86	.72	.36	.85	1.01	6.82
Bovine R	2.61	.60	.82	1.15	2.78	7.69
"t" value	10.8 *	2.2	5.1 *	2.9 *	10.4 *	7.1 *

* Statistically significant (P = .01).
g % = Absolute value for g of protein/100 ml serum based on total protein.

TABLE II. Electrophoretic Distribution of Protein Bound Polysaccharide (Glycoprotein) in the Rabbit, Guinea Pig and Rat following Tuberculous Infection.

Group	Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	Total polysaccharide
	mg %					
Normal rat	28.5	39.8	25.4	35.2	34.1	165
H 37Rv	33.8	32.4	47.0	40.9	31.1	185
"t" value	.9	1.2	6.8*	1.1	.6	8.7*
Normal rabbit	15.2	16.2	18.9	17.4	20.2	88
H 37Rv	23.0	14.2	24.8	28.4	30.5	121
"t" value	5.3*	1.1	5.6*	6.1*	6.8*	26.2*
Normal guinea pig	20.1	9.7	32.2	29.3	28.5	120
H 37Rv	29.4	20.3	55.9	40.7	32.7	179
"t" value	6.3*	9.2*	5.8*	4.7*	1.3	23.2*
Normal rabbit	15.2	16.2	18.9	17.4	20.1	88
Bovine R	28.0	20.9	25.2	31.0	48.0	153
"t" value	6.2*	4.3*	4.6*	5.8*	7.1*	25.2*

* Statistically significant (P = .01).
mg % = Absolute mg % protein bound polysaccharide/100 ml serum, calculated from total serum polysaccharide and relative distribution in protein fractions.

by electrophoresis. The significance of deviations from normal means was computed by the "t" test(9).

Results. Distribution and concentration of serum protein and glycoprotein before and after tuberculous infection in the rat, rabbit and guinea pig are presented in Tables I and II.

Protein. A significant hyperproteinemia occurred in infected guinea pigs and rabbits, but not in rats. Comparison of individual protein components before and after tuberculosis infection reveals several types of changes with respect to animal species and type of infection (Table I).

Glycoprotein. All 3 species exhibited increases in total serum polysaccharide following infection. Differential responses in the various animals were noted following tuberculous infection (Table II). Elevation of the polysaccharide content of the fractions also remained significant when expressed as milligrams polysaccharide per gram of protein.

Discussion. A previous study(6) revealed similar elevation of the total serum protein and gamma globulin in guinea pigs and rabbits, but not in rats, following BCG immunization.

The elevation in total polysaccharide appeared to parallel the degree of gross pathology in the tuberculous animals. The group showing the greatest increase in polysaccharide (rabbits infected with bovine type tubercle bacilli) also demonstrated the most extensive tuberculosis.

Increase in alpha-2 globulin glycoprotein seen in all animals following tuberculosis has also been described by Seibert(10) in clinical tuberculosis. Seibert's study suggests a possible correlation of elevated alpha-2 glycoprotein and increased tissue destruction. Our findings did not reveal this relationship in experimental tuberculosis. The greatest increase, for example, occurred in the rat, which had no gross pathology. The rabbits infected with bovine type bacilli had gross involvement of lungs, liver, spleen and kidney; whereas the rabbits infected with human type tubercle bacilli had only minimal involvement of the lungs. Despite these marked differences in gross pathology, the increase in alpha-2 glycoprotein was essentially identical in both groups. The guinea pig, with more extensive tuberculosis than the human strain infected rabbits and less pathology than the bovine type infected rabbit, had a greater alpha-2 glycoprotein increase than either of the two groups of infected rabbits.

Summary. 1. Total serum protein, polysaccharide and paper electrophoresis of protein and glycoprotein were determined before and 4 weeks following, tuberculous infection of rats, rabbits and guinea pigs. Hyperproteinemia occurred in infected rabbits and guinea pigs. Elevation of alpha-1 globulin in the guinea pig and alpha-2 globulin in rabbits and rats was noted. Beta globulin decreased in infected rats and increased in guinea pigs, and bovine infected rabbits. Gamma globulin elevated in all species. 2. Total serum polysaccharide was elevated in all infected animals. Likewise, the alpha-2 glycoprotein was elevated in all species. Only the infected rabbit had elevated gamma globulin glycoprotein.

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Amino Acid and Carbohydrate Composition of the Mucoprotein Matrix in Various Calculi.* (23161)

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A technic for recovery of the organic matrix from calcigerous urinary calculi has been described(1). Preliminary studies of the amount, elemental composition and some histochemical characteristics were reported(1,2). This report is concerned with the carbohydrate and amino acid content of matrix recovered from calcium containing urinary and

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submaxillary gland calculi and from urinary calculi of essentially pure uric acid and cystine composition.

Methods. The matrix from calcigerous urinary and submaxillary calculi was recovered by decrystallization with ethylenediaminetetraacetate, dialysis, ultrafiltration and lyophilization as previously described(1). Uric acid and cystine calculi were decrystallized in 0.1 M 5,5-diethylbarbituric acid buffer (pH 8.6) containing 10% neutral formalin as a preservative. Decrystallization was adjudged complete when the supernatant solution gave a negative Folin's test for uric acid or cyanide test for cystine. The recovery of matrix from the buffer solution was in all other respects similar to the procedure for calcigerous stones. *Amino acid components.* Samples of the lyophilized matrix were hydrolyzed in 6 N HCl for 24 hours at 90°C, blown dry, and further desiccated *in vacuo* overnight. This residue was taken up in a disodium versenate (0.5% w/v)-*iso*propanol (10% v/v)-water solution and centrifuged. The supernatant solution was chromatographed in 2 dimensions in each of the following solvent systems: (a) 2,6-lutidine:water (65:35 v/v) and phenol:water (4:1 v/v); (b) *n*-butanol:acetic acid:water (4:1:5 v/v) and *n*-butanol:ethanol (95%):water (4:1:1 v/v); and (c) *n*-propanol:ethyl acetate:water (7:1:2 v/v) and *iso*-butyric acid:water (4:1 v/v). Whatman #1 paper was used. Solvent ascension was begun after a 24-hour equilibration period. A unidimensional set of known amino acids was included in each direction with each group of unknowns. After 40 to 50 hours of migration the chromatograms were developed by spraying with 0.25% ninhydrin in acetone. The spots were marked as they appeared and identified by comparison of the Rf values with those of the knowns. Microbiological assays for amino acids were made on two samples of pooled matrix by the method of Henderson and Snell(3). *Carbohydrate components.* The technics of Glegg and Eidinger(4) were used for a 48 hour resin hydrolysis of matrix samples and unidimensional chromatography of the sugars. A double or triple ascension of 40 cm was made in each of the following solvent systems: (a) 2,6-lutidine:water (65:35 v/v); (b) *n*-butanol:acetic acid:water (4:1:5 v/v); and (c) *n*-propanol:ethyl acetate:water (7:1:2 v/v). Known sugars were run side by side with the unknowns. Studies with the diphenylamine reagent were made after the method of Winzler(5) utilizing standards of both crystalline "sialic acid" derived from human meconium† and 2-deoxyribose-5-phosphate.‡ Naphthoresorcinol tests for hexuronic acids were made after hydrolysis either with resin(4) or by the DeFrates-Boyd procedure. Controls were material isolated from urine by the DiFerrante-Rich technic(6) and commercial chondroitin sulfate. All spectrophotometric measurements were made with the Beckman DU instrument. Tests for lipids were made by the method of Artom and Fishman(7).

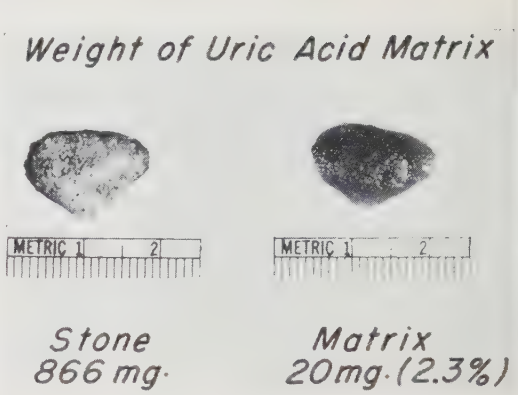

FIG. 1. Appearance and dry wt of typical uric acid stone before and after decrystallization.

Results. The organic matrix was distributed from the center to the surface of all calculi. In the majority of calculi the decrystallization left the matrix as a cast of the original stone. This was most apparent in the uric acid stones where the only visible evidence of decrystallization was a change in color from tan to greenish-brown (Fig. 1). In cystine stones the matrix usually collapsed into a disorganized sediment on decrystallization. The matrix accounted for 2.5% of the original dry weight of calcigerous stones, 2.3% of uric acid stones, and approximately

† Through the courtesy of Dr. Richard J. Winzler, Univ. of Illinois College of Medicine.

‡ Prepared by Dr. Sam. H. Love, Dept. of Microbiology, Bowman Gray School of Medicine.

TABLE I. Amino Acids Identified Chromatographically.

α-Alanine	Glycine
Glutamic acid	Threonine
Aspartic acid	Lysine
Serine	Proline
Leucine	(Tryptophane)*
Isoleucine	(Tyrosine)
Valine	(Methionine)
Phenylalanine	(Arginine)

* Amino acids in parentheses were faint and inconsistently seen.

9.0% of cystine stones.

The amino acids detectable by chromatography are presented in Table I in the descending order of visual intensity after ninhydrin development. Two or more unidentified ninhydrin-positive spots were consistently present in all matrix material. Their Rf values were not consistent with those of any commonly encountered amino acids. The severity of the hydrolysis should eliminate the possibility of any residual peptides in this material, but undoubtedly prevented the detection of certain compounds (*e.g.*, hexosamine). No differences were noted among the amino acid composition of any of the various matrix preparations regardless of the source or crystalline composition of the stones.

The results of microbiological assay of 17 detectable amino acids are given in Table II, with similar data for osseomucoid(8).

Chromatographically, 5 carbohydrates were clearly separated from the matrices of all calcigerous and cystine calculi, of both urinary and salivary origin. These migrated identically with the standards in each of 3 solvent systems and are the following, in the approximate order of their color intensity after treatment with aniline hydrogen oxalate: galactose, glucose, mannose, rhamnose, and fucose. In no instance has glucose been demonstrated in any pure uric acid calculus. This does not preclude its presence but does indicate that glucose is not present in uric acid matrix in the same concentration relative to other carbohydrates as it is in the other matrices.

Deoxypentose appears to be consistently present in small quantities in matrices from all calculi except that of cystine stones, which have not been thoroughly examined. This is based on the appearance of a pure blue color with the diphenylamine test for "sialic acid" (Fig. 2). This reaction is closely analogous to that described by Dische(9), and gives no color or different and weak colors, with other sugars so tested. Furthermore, the blue color developed in identically the same manner in both matrix and 2-deoxyribose solutions during the heating period of the test. The ultraviolet absorption curve gave no evidence of the presence of desoxyribonucleic acid in calculous matrix which gave the blue color reaction.

Quantitative assays of hexosamine and protein-bound hexose have given varying results with various pools and methods. The reason for these variations is being further studied.

Fig. 1 clearly demonstrates that "sialic acid" is not a component of matrix material. The tests for hexuronic acid were all negative although commercial chondroitin sulfate and material isolated from urine by the Di-Ferrante-Rich technic(6) gave positive results. There was no evidence of any cholesterol or lipides in a calcigerous matrix examined. A test for ketohexoses(10) was also negative in each of 3 calcigerous matrix pools.

Discussion. The finding of apparently the

TABLE II. Results of Microbiological Assay of Calcigerous Stone Matrix.

	Osseo-mucoid*	Stone matrix Range	Mean
Alanine	3.70	2.4 - 4.90	3.65
Glycine	2.65	2.1 - 3.26	2.82
Valine	4.50	1.8 - 2.12	2.11
Leucine	7.27	3.41 - 8.47	5.39
Isoleucine	3.65	2.23 - 4.00	2.88
Proline	4.24	1.10 - 2.11	1.53
Phenylalanine	2.86	2.11 - 2.46	2.29
Tyrosine	1.98	1.60 - 1.88	1.75
Tryptophan		.43 - .74	.53
Serine	3.61	2.70 - 5.36	4.07
Threonine	4.13	4.21 - 4.75	4.40
Cystine	1.13		
Methionine	1.09	.91 - .98	.95
Arginine	3.87	3.05 - 4.08	3.60
Histidine	2.65	1.19 - 1.52	1.30
Lysine	4.26	2.60 - 5.28	4.05
Aspartic	9.66	3.76 - 5.40	4.08
Glutamic	11.67	5.87 - 6.45	6.10
Total	72.92	41.47 -63.76	51.50

* From reference 8.

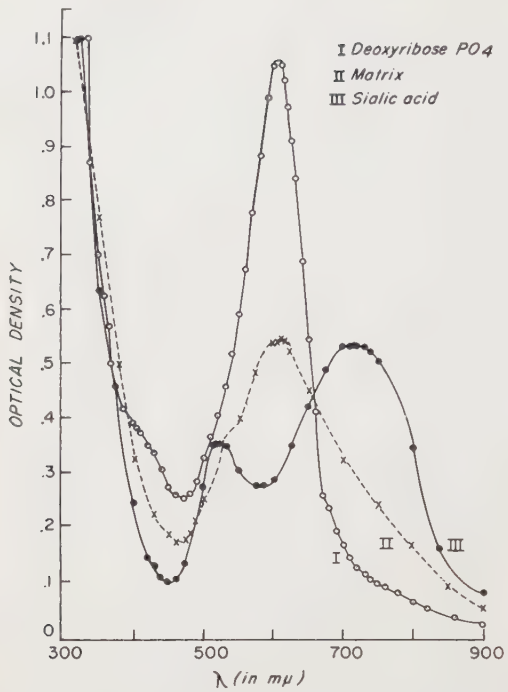

FIG. 2. Absorption curves obtained with 400 μg 2-deoxyribose-5-phosphate/ml (I), calcigerous stone matrix (II), and 100 μg crystalline sialic acid/ml (III) after reaction with diphenylamine. Each set on a blank without diphenylamine.

same spectra of amino acid and carbohydrate composition in the organic matrix of stones obtained from the ducts of the submaxillary gland and calcium containing stones in the urinary tract is of interest since the crystalline composition of stones from these sources is indistinguishable. Histochemical studies have previously indicated that matrices of both calcigerous urinary calculi and salivary gland calculi are PAS (periodic acid-Schiff leucofuchsin reaction) positive and also metachromatic with toluidine blue. Indeed, all urinary calculi are PAS positive, while metachromasia has not been observed in matrix from uric acid stones. It should be noted, however, that we have never seen a cystine calculus that did not show a faint opacification on clinical roentgenograms. Presumably this is due to a small amount of calcium bound in some way to the crystal surfaces. The absence of metachromasia in uric acid stone matrix may be related to the apparent absence of glucose. That is, the spatial configurations responsible for metachromasia in the mucoprotein may be related to the presence of the glucose molecule. There is no present evidence to indicate whether the glucose is related to the deposition of uric acid crystals or exclusion of calcium crystals from the matrix.

The demonstration that stone matrix is both PAS positive and metachromatic with toluidine blue has suggested the presence of both a neutral mucoprotein and an acid conjugated mucopolysaccharide. The fact that metachromasia is highly variable in many calculi and that it is necessary to study some serial sections of calcium containing stones before it is demonstrated seemed to bear out this supposition. If the metachromatic material is a mucopolysaccharide it is apparently lost in the dialysis procedures which are used to remove the decalcifying agents because we have found no hexuronic acid in the ultrafiltrate. Thus we have been able to obtain only a mucoprotein from stone matrix and it seems likely that the metachromasia is due to some specific configuration of a neutral mucoprotein which also gives a positive PAS reaction.

The occurrence of deoxypentose in this material is not proof that this material is an integral component of the carbohydrate moiety of the mucoprotein molecule. This sugar is presumed to be deoxyribose(11) since this is the only deoxypentose commonly encountered in human metabolism. Material isolated by the DiFerrante-Rich procedure (6) gives the same color reaction and is dialyzable (as is part, at least, of the hexuronic acid). The fact that it is present in matrices from both salivary gland and urinary calculi, is further indication that the matrix of these calcigerous stones is not a chance inclusion in a crystalline precipitate but a necessary part of calculus formation.

Summary. The amino acid and carbohydrate components of the organic matrix of stones from the submaxillary gland and from the urinary tract have been studied by paper chromatographic, microbiological and spectrophotometric technics. The chromatographic amino acid pattern was indistinguishable and

deoxypentose, hexosamine, and 5 other sugars were present in all of these matrices. The carbohydrate composition was similar in all stones with the exception of uric acid calculi, in which glucose appears to be absent.

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Metabolism of Steroids II. Half-Life of Various Steroids in Dogs.* (23162)

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Concentrations of free adrenal steroids in peripheral circulation are governed by a number of unknown factors, including rates of production by the adrenal cortex, of conjugation with other compounds, of excretion by the kidney and of *metabolism* in tissue. It has been established(1-3) that the disappearance of exogenous steroids from peripheral circulation is a first order reaction, *i.e.* rate of disappearance is proportional to plasma concentration. Accordingly, when logarithms of steroid concentration are plotted against time, a straight line results. From the equation for this regression line, the half-life of the steroid can be calculated. In human subjects the half-life of cortisol varies with age (4) and is influenced by certain diseases (1,2). Also, different steroids have different half-life values, but maintain a fairly consistent relationship to one another when studied in the same human subject(2,5). In the present study the rates of disappearance from the peripheral circulation of several different steroids have been measured in the dog.

Materials and methods. The animals used were trained, unanesthetized adult mongrel dogs, each weighing approximately 20 kg. Each animal was rested for at least one week and occasionally for several weeks between successive experiments. Thus, although each dog was used for many experiments during a 2-year period, and although blood loss per experiment approximated 200 ml, no animals developed anemia and the hematocrit values remained constant throughout an experiment. The steroids employed were cortisol (Compound F), cortisone (Compound E), corticosterone (Compound B), and Δ^1-cortisol (Δ^1-F). These compounds were prepared for intravenous administration by dissolving the free steroid in 50% ethanol.§ This solution was diluted in each case 15-20 fold with 5% dextrose in water and administered by intravenous drip during a 10 minute period. The amount of steroid given in each experiment was 2 mg/kg body weight. The moment the infusion was completed, was re-

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† A. H. Robins Post-Doctorate Research Fellow.

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§ Alcoholic solutions of these steroids were supplied through the courtesy of Dr. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich.

TABLE I. Half-Life of Various Steroids in 5 Dogs.

	Half-life (min.)				
	Cortisol (F)				
No. determinations	Mean & S.E.	Range	Cortisone (E)	Corticosterone (B)	Δ^1-cortisol (Δ^1-F)
25	51 ± 2.2	28-72	51	57, 30, 28	65, 56
26	49 ± 2.3	30-88	41	30	48, 56, 64
23	55 ± 1.8	44-81	38, 48	38	62, 73
6	64 ± 3.6	50-76		26, 35	114
4	51 ± 5.7	43-68		29	67
All dogs					
No. determinations	84		4	8	9
Mean ± S.E.	52 ± 1.2		45 ± 3.0	34 ± 3.5	67 ± 6.3
Range	28–88		38–51	26–57	48–114
	F		E	B	Δ^1-F
p *vs* F			<.50 >.10	<.01	<.01
vs E				<.10 >.05	.05
vs B					<.01

corded as zero time. Heparinized blood samples were obtained at intervals thereafter and the precise time of obtaining each sample was recorded; at least 5 samples were taken at 15 to 30 minute intervals. These samples were centrifuged after collection, and the plasma was separated and frozen until used for determination of steroid concentration. The concentration of Compound B was determined by modification of the fluorometric method of Sweat(6,7). Compounds F, E and Δ^1-F were determined as 17-hydroxycorticosteroids (17-OHCS) according to the method of Nelson and Samuels(8) as modified by Eik-Nes (9). Studies reported from this laboratory (10) have shown that this method can be used to determine Δ^1-F. In calculating the half-life for each of these steroids the logarithms of plasma steroid concentrations were plotted against time and the straight line of best fit was calculated according to the method of least squares. From the equation for this regression line the half-life of the steroid was calculated. Although evidence has been presented(11,12) that administration of cortisol and related steroids inhibits the release of endogenous ACTH, the duration of this action is not known. Hence, to minimize the influence of endogenous ACTH-induced corticosteroid secretion, plasma samples containing steroid concentrations less than 10 μg% were excluded from calculations of half-life. Physiological levels in dogs are well below this value(13).

Results. Our data are presented in Table I. The mean half-life for cortisol observed in 84 determinations in 5 dogs was 52 ± 1.2 minutes, with a range of 28 to 88 minutes. As shown in the column captioned "range," considerable half-life variation occurred among serial studies in each animal. Despite this, analysis of variance indicates that the probability of differences among these mean values being due to chance is less than 2%. Therefore, it appears that although unknown factors may influence this rate from day to day, the individual animal tends to remove free, exogenous cortisol from circulation at a characteristic rate and that this rate differs among different animals.

In the Table the half-life values for cortisone, corticosterone and Δ^1-cortisol in the same animals are compared with those for cortisol. The mean half-life for cortisone observed in 4 determinations was 45 minutes. This is essentially no different from that for cortisol. The value for corticosterone is much shorter than that for cortisol, with a mean of 34 minutes resulting from 8 determinations. The rate of removal from circulation of the synthetic steroid, Δ^1-cortisol, is definitely slower than those for compounds B, E, or F: the mean Δ^1-F half-life observed in 9 determinations was 67 minutes. In the 3 dogs in which more than one Δ^1-F half-life study was done not much variation was found, even though the range for the entire group was considerable (48-114 minutes).

Although the number of these Δ^1-F studies is too small to permit a definite conclusion, it appears that there is a tendency for the cortisol and Δ^1-cortisol half-lives to run parallel in the individual dogs: those animals which have a longer mean cortisol half-life also have a longer Δ^1-cortisol half-life.

Discussion. In these dogs the order for the rate of removal of exogenously administered steroid was Δ^1-cortisol < cortisol $\cong$ cortisone < corticosterone. This same order has been found in human subjects, but the 2 species differ with regard to the absolute half-life values for these steroids. Considering the 4 steroids in the order listed, the mean half-life values for dogs were 67, 52, 45 and 34 minutes, respectively; compared with these are half-life values reported for human subjects of 192(10), 106-115(1,2), 94(14) and 39 (14) minutes, respectively.

Proportionate rates for disappearance of exogenous steroids also are different in the 2 species. Thus, the ratio for the mean half-life of each steroid in man to that observed in these dogs is roughly: for Δ^1-cortisol, 3; for cortisol, 2; for cortisone, 2; and for corticosterone, 1. For each steroid except corticosterone the half-life in the dog is much shorter than that in man. This may explain partially why the dog has a much lower plasma 17-hydroxycorticosteroid concentration than man(13) even though in both species cortisol is the major corticosteroid secreted by the adrenal cortex.

The relationship of Δ^1-cortisol half-life to cortisol half-life has been discussed elsewhere (10,15). In the metabolism of Δ^1-cortisol it first is converted to cortisol by reduction of the C^1 double bond before further saturation of ring A at C^4, and subsequent hydrolysis of the C^3Ketone(16,17). On the basis of the smaller Δ^1-cortisol/cortisol half-life ratio in dogs (1.3) than in man (1.8), there is indication that C^1 reduction occurs more rapidly in the dog.

Summary. Data concerning the half-life of exogenous cortisol, cortisone, corticosterone and Δ^1-cortisol in dogs were presented. Cortisone half-life was similar to that for cortisol. In contrast, Δ^1-cortisol half-life was considerably longer and corticosterone half-life considerably shorter. The relationship between these findings in dogs and those in human subjects was discussed.

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Pharmacological Studies of a New Oral Hypoglycemic Drug. (23163)

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In the past 25 to 30 years, a number of synthetic hypoglycemic drugs, for oral as well as parenteral use, have been described. None have proven successful in the treatment of diabetes. Slotta and Tschesche(1) reported on the oral hypoglycemic action of some lower alkyl derivatives of biguanides (referred to here as formamidinyliminoureas). A systematic investigation was, therefore, undertaken involving the synthesis and evaluation of approximately 200 new mono- and disubstituted alkyl and aralkyl derivatives of formamidinyliminoureas.

The present report deals with the pharmacological properties of one of these derivatives, N′-beta-phenethylformamidinyliminourea HCl (DBI) which was found to be a highly active oral hypoglycemic agent in both normal and alloxan-diabetic animals.

Method. DBI, a water-soluble, crystalline compound, was given in solution by stomach tube unless otherwise stated. Blood samples were collected from animals fasted for 18 hours. *Blood sugar* was estimated by micromodification of the Folin-Wu method and expressed in mg/100 ml of blood. Glycogen was estimated by the method of Good, Kramer and Somogyi(2). *Diabetes* was produced by administration of alloxan; rabbits received 200 mg/kg intravenously, rats 250 mg/kg intraperitoneally and Rhesus monkeys 150 mg/kg intravenously. Rabbits and rats were used 5 to 7 days after injection. One monkey in which diabetes developed, reached a stable high blood glucose level 2 weeks after alloxan administration.

Results. Table I summarizes the results obtained in guinea-pigs, rats, rabbits, cats

TABLE I. Action of DBI on Blood Sugar in Normal Animals.

	Dose, mg/kg	Blood sugar, mg/100 ml, hr						No. of animals
		0	1	2	3	5	24	
Guinea-pigs, subcut.	5	74	68	66	69	63		4
	10	88	75	40	25	42		4
	20	85	88	33	17.5†	*		4
	28	82	94		10			4
	33	84	90		16	22		4
	65	79	7		*			4
Guinea-pigs, oral	20	83			80	56	86	4
	25	89			69	39‡	86	15
	30	83	79		62	34†	82	4
	40	75			47	20†		4
	50	93	100		29	17*		6
Rats (CFW strain), oral	50	72			75	65		4
	80	75			70	56		4
	120	68			56	32	66	4
Rabbits, oral	20	80			88	62	85	3
	40	88			66	45	82	4
	60	79			44	28†	76	4
Cats, oral	20	60				52		2
	40	70				39		2
	50	72				16*		2
Rhesus monkeys, oral	2	62			60	57		2
	5	63			53	46		3
	10	59			43	25	62	4
	15	66			36	12	58§	4
	25	58			5	*		2

* All died. No glucose given. † Two died. No glucose given. ‡ Five died. No glucose given. § Two out of 4 animals were given glucose between 5th and 7th hr.

and monkeys. After observing that DBI lowers blood sugar when given subcutaneously to guinea-pigs, further testing was done by oral administration. It is seen that hypoglycemia reached its maximum in 5 hours, and in all cases tested it was back to normal in 24 hours. The species most sensitive to the drug is the Rhesus monkey, followed by guinea-pig, rabbit, cat and rat. Variation was high even within the same species. DBI was tried in dogs, in which it failed to cause hypoglycemia, but produced a complex effect which will be reported later. Death by hypoglycemia occurred in a number of animals of all species investigated. This could be prevented by prolonged administration of glucose.

In alloxan-diabetic animals it was possible to bring the blood sugar to normal levels by appropriate doses of DBI (Table II). The correct dosage was reached by gradual increase in the daily dose, starting with the dose which was 50%-effective in normal animals. When the normal level was attained, the animals were maintained with the effective dose for 3 days in rats and 5 days in rabbits. In 2 cases, where animals required high doses of DBI, they died before normal blood sugar values could be reached. When DBI-treatment was discontinued, the blood sugar values rose slowly and reached pretreatment level in 4 to 7 days.

Fig. 1 shows effect of DBI on the alloxan-diabetic monkey. This animal was treated for 26 days with oral administration of 10 mg/kg, later reduced to 9 and 8 mg/kg. Under the influence of treatment the blood sugar dropped from 228 mg % to normal and once to 16 mg %. The hypoglycemia was reversed by administration of glucose. It is seen that suspension of treatment always resulted in hyperglycemia which was promptly corrected by resumption of DBI administration.

TABLE II. Action of DBI on Blood Sugar in Alloxan-Diabetic Animals.

	Initial blood sugar*	Daily dose, mg/kg	Final blood sugar*
Rabbit	232	60	60
	177	50	66
	188	40	75
	418	200	200†
	190	90	95
	189	90	85
	198	100	98
Rat (CFW strain)	194	500	83
	326	1000	102†
	192	400	75
	161	150	82
	320	500	70
	257	1200	76
	265	450	87
	174	100	66

* mg/100 ml.
† Died before normal level could be reached.

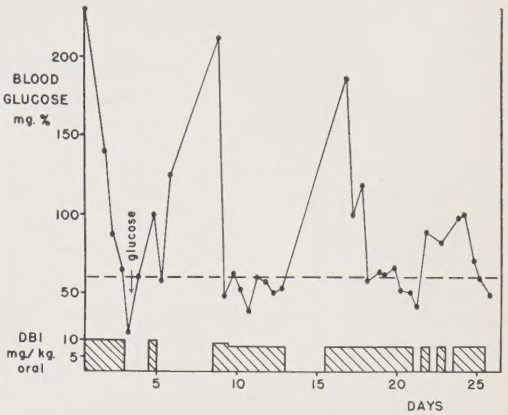

FIG. 1. Effect of oral admin. of DBI on blood glucose in alloxan-diabetic Rhesus monkey. Abscissa, time in days; ordinate, blood sugar levels in mg %. Broken line indicates avg blood glucose in normal monkeys. Dosage indicated at bottom of chart in mg/kg/day given in single oral admin. Blank spaces indicate intervals (usually weekends) when DBI was not admin.

The effect of DBI on liver and muscle glycogen is shown in Fig. 2. Five hours after administration there was a slight fall in liver glycogen (31%), not more pronounced than that observed after insulin. Twenty-four hours after treatment both liver and muscle glycogen levels were higher than in the controls. In alloxan-diabetic rats there was a marked glycogen increase in both liver and muscle 5 hours after treatment with DBI. The effect on glycogen, however, should be interpreted with caution because of the extreme variability of glycogen levels in control animals.

Evaluation of toxicity of DBI is made difficult by the fact that the animals die of hypoglycemia before any other toxic effects can be observed. Thus the LD_{min} in mice injected intraperitoneally was 100 mg/kg and the oral LD_{50} in guinea-pigs 40 mg/kg. Experiments

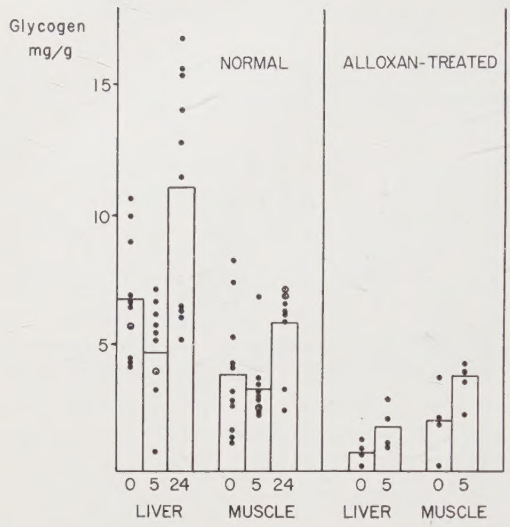

FIG. 2. Liver and muscle glycogen changes after oral admin. of DBI to normal guinea-pigs (20 mg/kg) and alloxan-diabetic rats (500 mg/kg). Abscissa: time in hr after admin. of the drug; ordinate: glycogen in mg/g.

now being conducted show that, under administration of glucose, the animals can tolerate very high doses of DBI. The results obtained in alloxan-treated animals also show that they can tolerate several times the median hypoglycemic dose of DBI without toxic effect. Two alloxan-diabetic animals died without evidence of hypoglycemia (Table II). At the high doses of DBI these animals were receiving, the toxic effect may have been independent of hypoglycemia. Ten guinea-pigs receiving 20 mg/kg of DBI orally for 2 weeks showed no untoward effect on their blood chemistry (other than a fall of blood sugar to 40 to 50 mg %), blood cells and coagulation. Pathological examination of tissues from these guinea-pigs and from a monkey which died as a result of hypoglycemia after a high initial dose of the drug (25 mg/kg), showed no damage in kidney, adrenals, liver, spleen, intestine, heart, pancreas, skeletal muscle, lungs, testes, lymph nodes and thymus.

Outside of its hypoglycemic effect, DBI has no marked acute pharmacological actions; at high doses in rats it has a slight depressive action on spontaneous motor activity and has a mild antidiuretic effect. On intravenous injection into dogs, it causes a transient rise of blood pressure; it has no potentiating or blocking action on epinephrine, acetylcholine or histamine.

Discussion. The hypoglycemic agent described above is different from other orally active drugs hitherto described. Under the conditions of our experiments, it did not cause significant and durable glycogen depletion. Results of pathological examination also show that no histologically detectable liver damage was produced by acute administration of a high dose or prolonged administration of what may be called a therapeutic dose. Unlike the sulfonylurea derivatives recently investigated, DBI was fully active in alloxan-diabetic animals. It is therefore probable that it can act in the absence of insulin. Preliminary experiments, which will be reported, suggest that DBI increases glucose uptake in the isolated rat diaphragm. This would suggest that DBI may act by a mechanism involving peripheral glucose utilization.

Summary. N′-beta-phenethylformamidinyliminourea (DBI), a synthetic substance, administered parenterally or orally, causes hypoglycemia in guinea-pigs, rats, rabbits, cats and Rhesus monkeys. It can also reduce blood sugar in alloxan-diabetic rats, rabbits and monkeys and maintain them at a normal level. DBI does not cause significant changes in glycogen content of liver or muscle. Further work will be necessary to elucidate its mechanism of action and assess its possible clinical usefulness.

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Clinical Report of a New Hypoglycemic Agent. (23164)

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A wide variety of chemical substances of both natural and synthetic origin is known to effect reduction of glycemia on oral administration(1). During the past few years, investigators both here and abroad have demonstrated that oral administration of aryl sulfonylureas can substitute for exogenous insulin in mild, usually obese and elderly diabetic patients(2). The narrow range of applicability of these drugs has recently been stressed and, with some, severe toxic reactions have been reported. The site and mode of action are obscure. The search for a safe, effective oral insulin substitute still continues. A new synthetic drug N′-beta-phenethylformamidinyliminourea (DBI)* unrelated to the aryl sulfonylureas has been evaluated in the laboratory animal. Given orally, it effectively lowers blood sugar levels in alloxan treated diabetic rats, rabbits and Rhesus monkeys. A hypoglycemic effect is also seen in normal animals on oral administration(3).

The present report deals with observations in man on the acute action of N′-beta-phenylethylformamidyliminourea on glucose tolerance. Short term observations are also reported on diabetic patients when insulin and/or the drug are alternately given and withheld.

Method. Ten adult patients with diabetes mellitus and one subject without any evidences of a disturbance of carbohydrate metabolism were studied. The subjects were fasted overnight before each test and those with diabetes were not given any insulin on the morning of the test. Venous blood samples were taken before and at hourly intervals for 6 hours after the patients drank a solution containing 100 g of glucose. The tests were similarly repeated 1 week later when 100 mg DBI was given with the glucose solution. Blood glucose was determined by the method of Folin-Wu. Severe labile, moderately severe and non-insulin diabetic patients were included in the study. Three diabetic patients were observed for 6-10 day periods during which blood and urinary glucose were determined twice daily and insulin and/or DBI were alternately given and withheld. A 27-year-old male with labile diabetes mellitus of 8 years duration, a 38-year-old female with diabetes mellitus of 12 years duration controlled with 22 units of NPH insulin, and a 68-year-old female with diabetes mellitus of 28 years duration are included.

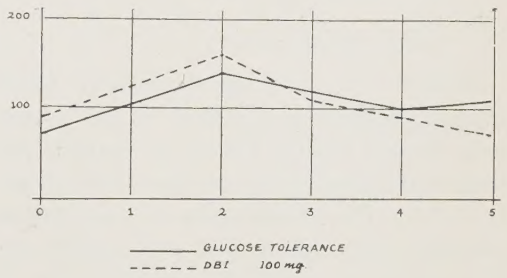

FIG. 1 (non-diabetic).

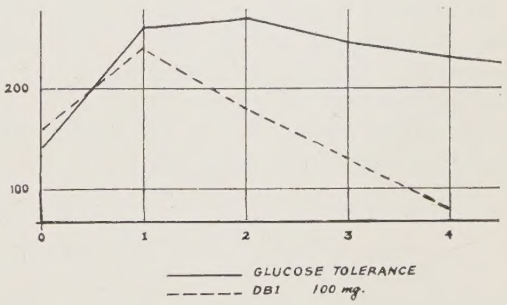

FIG. 2 (diabetic moderately severe).

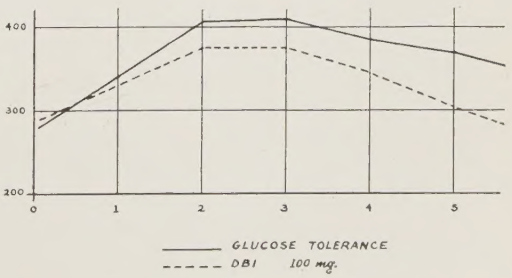

FIG. III (diabetic severe).

* We are indebted to Dr. L. Freedman, U. S. Vita. Corp., for generous supplies of DBI and the experimental data concerning it.

TABLE I. 10-Day Observation of a 27-Year-Old Male with Diabetes Mellitus; 8 Years Duration.

Days		9 A.M. B.S. (mg %)	9 A.M. U.S. (%)	3 P.M. B.S. (mg %)	3 P.M. U.S. (%)
1	NPH insulin, 70 units	184	trace	198	.25
2	*Idem*	168	"	202	.25
3	NPH insulin, 30 units	178	"	306	2
4	*Idem*	298	2.4	344	3.1
5	" & DBI, 100 mg t.i.d.	304	3	188	.25
6	*Idem*	156	0	180	trace
7	NPH insulin, 30 units; no DBI	158	0	244	1.5
8	*Idem*	306	3.5	385	4
9	NPH insulin, 30 units; DBI, 100 mg t.i.d.	311	4.2	264	1
10	*Idem*	166	0.25	144	0

Results. A significant decrease in blood sugar concentration occurred in the apparently normal subject and in the diabetic patients after ingestion of 100 mg of DBI (Figs. 1, 2, 3). The configuration of the glucose tolerance curve taken after oral administration of 100 mg of DBI was altered. This is unlike the action of sulfonylurea drugs which lower fasting blood sugar but do not alter the configuration of the glucose tolerance curve(4). Nausea and vomiting occurred during the fourth hour in one patient (Fig. 2) and required termination of the experiment. Blood glucose at this point was 80 mg %.

DBI adequately replaced 40 units of the 70 required units in a young severe labile diabetic patient (Table I) and totally replaced insulin in a less severe diabetic patient under 40 years of age and in a 68-year-old patient whose diabetes has been present 28 years.

Discussion. DBI effectively reduces blood sugar within 3 hours on oral administration. This effectiveness is maintained longer than 6 hours. The data reported herein suggest that DBI can be effective by mouth in decreasing the blood sugar of patients who develop diabetes before 40 years of age as well as those who have had diabetes more than 20 years. The data suggest that insulin requirement of patients with severe diabetes mellitus can be significantly reduced.

Conclusions. DBI by mouth produces an improvement in glucose tolerance in patients with severe as well as mild diabetes mellitus. It appears to be an active potent hypoglycemic agent, effective in patients with mild, moderate and severe diabetes mellitus. The results herein presented lend encouragement to wider experimental and clinical investigations.

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